

Mandela must look to the north

The burial of apartheid last week in South Africa (the practice ended some years ago) is a landmark in history, as Mr Nelson Mandela has been saying, but what some call the struggle is just beginning.

POLITICIANS whose reputation is enhanced by their engagement in a contentious election are few and far between, but Mr Nelson Mandela is one. His demeanour during the past few weeks has been as correct as in the five years since his release from jail. That he should be able to tell the *Guardian* newspaper, on the eve of his appointment as president of South Africa, that he wishes to "let bygones be bygones" is itself a monumental declaration. Not many who have suffered a quarter of a century of unjust imprisonment would readily rise to that. But a period of reconciliation is precisely what South Africa needs.

What are the chances of that happening? Imponderables are evenly balanced on either side. South Africa's strength is that it is the richest country in southern Africa, and populous as well. Its weakness is that extremists of all stripes are discontented with the way the elections have turned out, while Mandela's own supporters have good cause to be impatient for an improvement of their lot. One immediate task for the new government will be to ensure somehow that the satisfaction of these demands can be contained within the available resources. Another, and more important, is to negotiate and agree a constitution that will serve for the long haul (and which must be in effect five years from now). What with the need to keep the civil peace as well, even the lion Mandela will be asking whether that is not enough to be going on with.

But there is yet more to be done. South Africa is not merely the best endowed country in Africa south of the Equator, it is also the one best placed to help rescue the remainder of the continent from what at present appears to be a nasty future. Much can be done by example, which is why the permanent constitution of the republic could have beneficial consequences far outside its borders; at least one of the causes of the slough into which Africa as a whole is falling is the pattern of inefficient, intolerant and often corrupt government that appears to be endemic. If Mandela can pacify Kwa-Zulu interests, perhaps Nigeria (much further north) need not be tearing itself apart.

There is also technical work to do. For decades, research organizations in the republic have been saying that they could help to make the rest of Africa prosperous if only they were given the opportunity. In fields as different as agriculture, public health, mining technology and construction, government agencies in the republic have much in the way of advice to offer. Hitherto, the slur of apartheid has meant that their advice, let alone involvement, in development

outside the republic has been scorned. But last week's election should finally have put an end to that state of affairs, providing southern Africa as a whole with a source of now-indigenous expertise it often lacks. It would be a great misfortune for Africa in general if this opportunity were overlooked in the hectic months ahead. □

British research hiatus

An over-glossy statement of what the future holds leaves many important questions without answers.

TYPOGRAPHICALLY, the British government has done the scientific community of which it is the chief supporter a great honour by the lavishness of *Forward Look 1994*, the first of what is meant to be an annual series of publications charting the evolution of a new strategy for research. For one thing, the text appears to have been set in 9-point type on 15 points of lead, which means that white space would account for a large part of the typeset area on every page even if every character were a capital letter. The document is made even easier on the eye by the 7-cm margin on the outside of the first 50 pages of the text and generous space between paragraphs, and by the elegant copper-simulating colour used for headings in the text and for the interleaving pages that separate chapters from each other. Those who consider the price (£30.00 for two volumes, from HMSO) exorbitant for what is meant to be a working document will at least have a fine example of contemporary typographic design.

Sadly, the text does not live up to its appearance, either in literacy or in content. The British Civil Service, which some years ago gave the world the new English noun *spend* (as in "the annual spend will be...") has taken this opportunity to launch the novel noun *build*, which appears to signify the amount of building (physical or metaphorical) accomplished at some defined instant, and *secondees*, which can be taken from the context (the DTI is "increasing the number of industrial secondees within its Innovation Unit") to mean a person who has been temporarily transferred from industry to the public service. There is also a new gloss on the meaning of the noun *essay*; the documents include a series of writings so labelled, but misleadingly; they do not have the shape of essays (which advance a proposition and then set out to sustain it by literate argument) and which, by being anonymous, cannot be assigned intellectual weight (but the



over-boastful piece on biotechnology, "Building for the future", smacks of amateur copy-writing that may yet be employed in the advertising industry).

The content of the documents is inevitably diluted by the government's decision to wait for the outcome of its first exercise in "technological foresight", on which 15 panels and a steering committee are now labouring. The aim is to define fields in which the concentration of research is reckoned likely to contribute to "wealth creation"; the hope is that the process (involving the consultation of at least 7,000 members of the scientific public) will be complete by early next year, in time for *Forward Look 1995*. Meanwhile, the government's funding mechanisms are being polished and tuned to exploit the opportunities that lie ahead.

There is a lot going on. Provisionally, at least, the word "relevance" has crept back into the definition of the research that will in future be supported. The funding councils that support universities, for example, are warned that they must by next year have developed incentive schemes that "take into account the value of research that is of general relevance to industry and other users". And, while waiting for the outcome of the foresight exercise, the research councils (which make grants to individual researchers, among other things), will be expected to apply the criterion "innovation PLUS relevance".

It is too soon to know how all this will work. Much will hang on the outcome of the foresight exercise. If the strategies with which the research councils are then blessed are coherent and intelligently broad, the government's dreams may yet come true. The obvious danger is that Britain's academic researchers will be sent scurrying towards more immediate goals. One of the virtues of this year's document is its explicit promise that British researchers will "not be converted into short-term problem solvers for industrial customers". If that is so, one of the British research community's worst fears will have been exorcised. The promise will be remembered until experience shows that it is being kept.

There are two other immediate worries, of which the first stems from the element of directedness that will be unavoidable when research councils are given not merely "terms of reference" (as now), but also acquire explicit strategic goals. It seems to be agreed that the scientific merits of grant proposals will be judged by peer review, but who will judge whether proposals considered sound are likely also to contribute to a research council's strategic goals? There is nothing in *Forward Look 1994* to suggest that the government yet appreciates the intellectual difficulty of that task.

The second worry is more serious: money. The essence of the government's new policy for research is that more of it should be "relevant" research, but at no stage has it been considered that more relevant research may also be more expensive. Yet that is the common experience (and the conclusion of the Gibbs-Zuckerman study published by the British government 35 years ago). Is the British government prepared to meet the extra costs or will the result be a further concentration of research on fewer projects at fewer institutions? And what will be the consequences of that for the output of skilled people on whom the future hangs? □

Spying is bad business

Why should there not be international restraints on the distasteful espionage business?

THE imprisonment last week of Mr Aldrich Ames, the official of the US Central Intelligence Agency who became a Soviet and then Russian spy, comes distastefully on the heels of the book by Pavel Sudoplatov, the Russian spymaster who claims to have organized the flow of espionage material about nuclear weapons from the United States to the Soviet Union (see *Nature* 368, 779; 1994). Ames pleaded guilty to charges of having sold information about the identity of US intelligence agents who were Soviet nationals, many of whom were then executed. Sudoplatov's tale is also rich in its account of personal responsibility for the death of other people, including one he killed with a bomb planted with his own hands.

Killing people is generally accepted to be wrong. The exceptions to this moral rule are those arising when the killers and the killed are engaged in war. There are even formal international agreements (the Geneva conventions) defining the conditions that must then be satisfied — those concerned must be wearing distinctive uniforms, for example. Sudoplatov in his book is evidently troubled on this score, repeatedly declaring that he believed he was engaged in a war against enemies of the state employing him. Ames seems to have had no such qualms. The subversion of people's loyalty from the communities to which they belong is just as corrosive of civility as outright murder, and much more common.

It is in everybody's interests that these practices should be constrained, even stopped. But would that not imply an end to espionage, which is impossible? Not necessarily. It is entirely proper that national governments should try to understand what other governments are seeking by their policies to accomplish, so that collecting information about fellow (especially rival) governments is legitimate. It also now seems to be accepted that information gathered by Earth observation satellites or from electromagnetic signals picked up electronically are legitimate grist for the intelligence mills, while embassies in foreign capitals are probably now more useful than they have ever been. So the need for old-fashioned spies should be declining. The chances are that it is not.

What restraints could there possibly be? Most obviously, an international non-governmental organization could, by monitoring and making public what is known about current espionage, be both a public service and a restraint. (Amnesty International has done wonders for human rights.) Better still would be an international agreement among governments that the subversion of others' nationals by espionage would be punishable by the payment of compensation — say one per cent of gross national product per spy. That might not halt the sordid business, but even the richest governments would be given pause. Ideally, they might then be ready to license all spies, so rendering them ineffective. □

Russian mission to Mars expected to be held back for two years

Washington. Russian managers of the Mars 94 planetary mission are reported to have given up hope that the spacecraft can be launched in October as planned and are expected to announce soon that the mission has been delayed until 1996.

Planning for the Mars 94 project, which began in the 1980s and eventually involved scientists from 20 countries, would have sent an orbiter, two small landers and two surface penetrators to investigate Mars. Since then, however, the project has fallen victim to financial, technical and scheduling problems.

Last year, funding by the Russian government was held up for so long that spacecraft builders could not pay their subcontractors, and work slowed down. By the time the money was restored at the end of the year and work resumed, "the schedule had zero contingency in it", according to Louis Friedman of the Planetary Society, which is based in the United States.

There were technical delays as well, notably with the German-built Argus platform for pointing the spacecraft's cameras. Earlier this year, project managers considered launching in October anyway, even if all the instruments were not ready in time. Now, that plan has also been abandoned.

Instead, the launch will be postponed until the next Mars 'window' in 1996. Another Russian mission originally planned for that slot, to include a French-built balloon experiment and a rover vehicle for exploring the Martian surface, has been pushed to 1998.

But these plans may also unravel. The 1996 opportunity requires more launch energy than this year's, and this means that the Mars 94 instrument payload will probably have to be split into two launches, according to Roald Sagdeev, a former director of the Soviet planetary programme who is now with the East-West Science and Technology Center at the University of Maryland.

In such circumstances, he says, some of the instruments from the 1998 mission could fly in 1996, although the French balloon experiment may not be ready by then.

Friedman, whose society is involved in the 1998 balloon and rover experiments, believes the Russians will have enough money to launch some kind of Mars mission in 1996. "On the Russian side they may ironically have less of a problem in this regard than the Western partners," he says, as funding for Mars 94 has already been

committed by the Russian government.

In fact, one reason for delaying the official announcement of the mission's postponement has been to get as much work finished this year as possible. With inflation in Russia running at 10–20 per cent a month, funds appropriated last year buy less and less with each passing day.

But keeping engineering and science teams together for another two years is a more serious problem. Sagdeev estimates

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Still waiting: Martian volcanoes based on data from Viking mission.

that IKI, the Russian Space Research Institute, has already lost 20 to 30 per cent of its staff. If no more money is forthcoming from the government for space missions, engineers at the Lavochkin organization that builds the spacecraft, as well as its suppliers, would have to close shop and search for other work.

The only salvation for the Russian space programme may be in cooperation with the United States. Sagdeev and others hope that the Russian 1998 Mars mission can be coordinated with NASA's planned Mars Sur-

veyor series, which would encourage the Russian government to keep supporting it.

Cooperative agreements with the United States have already introduced substantial stability into the Russian space programme. Working groups between the two countries are discussing possible joint projects, which could include missions to explore Mars, Pluto and the Sun.

But, after 30 years, the era of Russian-led planetary missions appears to be drawing to

US Geological Survey/SPL

a close. At a recent symposium at Johns Hopkins University, Sagdeev detailed how things have changed for the once-exalted Russian space programme. Whereas the VEGA missions that visited Venus and Halley's comet in the 1980s cost only \$55 million for two spacecraft, the single-shot Mars 94 was priced at \$200 mil-

lion. Proton rocket launches that used to cost Russian scientists only \$2.5 million now go for 10 times that much.

Perhaps worst of all, says Sagdeev, the Russian space programme, stripped of its Cold-War aura, now has to compete with other disciplines for funding. After years of special treatment, he said, space scientists could be forced to go back to join the "traditional, conventional science community"; and that, he warned, would have a "tremendous negative impact".

Tony Reichardt

UK council backs peer review shift

London. Britain's new Engineering and Physical Sciences Research Council (EPSRC) has given its approval in principle to a more streamlined system for assessing grant applications.

Council officials say that the proposed changes, which they hope will receive final approval at the council's next full meeting in June, will reduce the amount of time and paperwork involved, as well as ensuring that research projects are more closely aligned with the stated 'mission' of the council.

But some scientists remain worried over the extent to which the changes will impose a heavy liability on programme managers who may have little first-hand experience of the research for which they are responsible.

Under the system operated by the EPSRC's predecessor, the Science and Engineering Research Council (SERC), grant applications were approved through a hier-

archy of peer-review committees. Smaller grants were awarded directly by discipline-based panels and subcommittees, but larger grants required the additional approval of committees sitting above them; grants worth more than £1 million required the approval of the whole council.

Two factors have determined the proposed change in strategy. One has been a desire, actively encouraged by the Office of Science and Technology, to reduce administrative costs associated with the review of grant applications, and increase the efficiency with which applications are handled.

The second has been the need to fulfil a requirement imposed by last year's white paper on science that the research council should explicitly commit itself to increasing Britain's industrial competitiveness (and maintaining the quality of life).

A paper prepared by EPSRC officials ►

and presented to council members at their first full meeting two weeks ago set out a strategy for addressing both issues by replacing the traditional system of peer-review committees with the new system.

Now, decisions on grant awards will be the responsibility of programme managers. Decisions will still be based on advice from scientific peer-review committees; but programme managers will also be expected to take into account input from other sources about how the research in question will meet the EPSRC's explicit mission.

The approach was agreed upon in principle by the council, although formal approval is being withheld until the council has been told how the new system will operate in practice — and, in particular, how the balance will in practice be struck between scientific and other criteria.

Officials of both the EPSRC and the OST have been taking pains to stress that the new system is not intended to replace the traditional peer-review system. William Waldegrave, the cabinet minister responsible for science, has said in a letter to the opposition science spokesman, Lewis Moonie, that peer review will remain a "key element" in determining the quality of basic science research proposals.

But Waldegrave also emphasized that grant applications will not be judged on scientific merit alone, and that attention will also be paid to the extent to which the research proposed is aligned with technological priorities identified, for example, by the Technology Foresight programme.

"The way that the SERC handled grant requests had certainly become very bureaucratic and time-consuming, with far too much micro-management by committees," says John Mulvey of the University of Oxford. "But if a lot of real responsibility is delegated to civil servants in the Swindon office [of the EPSRC], then many scientists will be watching this with caution."

EPSRC officials say that the new responsibilities of programme managers will be little different from those occupying similar positions in the US National Science Foundation (NSF). But British scientists point to the important difference that the US officials are frequently university scientists on secondment to the NSF, whereas their British counterparts are full-time civil servants.

Meanwhile, the Higher Education Funding Council for England (HEFCE) has published draft proposals of criteria it suggests using to allocate money to university departments to support "generic research" of potential interest to industry.

University departments will qualify for such extra funds, the HEFCE proposes, provided that they already receive research support of at least £20,000 a year from at least three different outside bodies.

The HEFCE has already decided that £10 million should be awarded to university departments for generic research in the next academic year. □

Scotland digs in over mooted shake-up of research labs

Edinburgh. Centuries of ill-feeling between England and Scotland have resurfaced over a proposal by a group of civil servants that a number of Scottish research institutes be grouped together into new agencies "owned" either by the London-based Ministry of Agriculture Food and Fisheries (MAFF), or the Biotechnology and Biological Sciences Research Council (BBSRC).

The proposal is contained in the "emerging findings" of a review of government research laboratories being carried out by the Cabinet Office's Efficiency Unit currently circulating in Whitehall. It is one of several proposals designed to rationalize the organization of these laboratories by grouping them according to common interests (see *Nature* 368, 681; 1994).

The preliminary conclusion of the group, for example, is that all government research institutes in the United Kingdom relating to the marine environment might be placed in a single grouping "owned" by the Scottish Office, while those relating to food production be reorganized under MAFF, and more basic biological research to the BBSRC.

But some Scottish scientists are worried that, if accepted by the government, such proposals could undermine what is described as the "Scottish system" of research support. This is based on encouraging strong vertical links between institutes involved in basic and applied research, as well as horizontal links between those involved in different areas of science.

Others fear that, although the amalgamation proposed in the preliminary conclusions might lead to a more cost-effective use of research resources for Britain as a whole, it could result in funds being taken away from smaller Scottish institutes as resources are concentrated on larger institutions south of the border.

"The problem is that, looked at on a UK basis, Scottish institutions that play a useful role locally can be totally outweighed by bodies in the south", says Maxwell Irvine, principal of the University of Aberdeen, which is currently involved in a scheme linking several local institutions — such as the internationally known Rowett Research Institute and Macaulay Land Use Research Institute — into a joint research consortium.

"We would be very concerned if those who are playing national games [with the reorganization of research institutes] were to weaken what we see as a very positive regional development."

The dispute has also highlighted an apparent difference in research strategies between the Scottish Office, which is keen to

play an active part in stimulating collaborative efforts by the private and public sectors, and the Cabinet Office in London, which is placing greater emphasis on reducing direct

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Fish farming: a new research-based industry.

public support for innovation.

Last year, the Scottish Office's Agricultural and Fisheries Department published a policy statement suggesting an expansion of the Scottish system under a Scottish Biological Sciences Board.

Some fear that, if implemented, the scrutiny team's proposals could introduce new vertical and horizontal distinctions between institutes that would cut directly across the Scottish Office's attempts to build an integrated system of research support.

Jean Balfour, a former chairwoman of the Scottish Countryside Commission, warns that such a move would be "extremely destructive to the whole question of how research is carried out in Scotland".

At the same time, the dispute has fanned a more widespread disenchantment in Scotland with some of the policies of the Conservative government, particularly since cuts in public spending have frequently hit those areas in which Scotland has benefited substantially from its membership of the United Kingdom in the past.

At a meeting in Edinburgh last month organized by the Royal Society of Edinburgh as part of the Edinburgh Science Festival, for example, Bruce Pattullo, the governor and chief executive officer of the Bank of Scotland, warned that privatization — one of the government's main policy objectives — "is about maximizing short term cash returns to the Treasury, and not about maximizing competition".

William Waldegrave, the cabinet minister responsible for science, has promised that the final version of the scrutiny team's report will be made available for public comment before any of its recommendations are acted on by the government. Several Scottish research institutes are said to be sharpening their pens in readiness.

David Dickson

Rees Features

OECD gives Japan mixed score on environment

Tokyo. Japan's efforts to preserve its environment over the past several decades have been both praised and criticized in a comprehensive review by the Organization for Economic Cooperation and Development (OECD). The report points out that over the past two decades Japan has achieved the fastest economic growth among advanced nations, but despite industrial success has shown the best performance among OECD countries in reducing emissions of a number of pollutants.

At the same time, however, the 210-page report focuses on a number of severe shortcomings of Japan's environmental performance. Waste-water treatment comes in for particular criticism. Only 44 per cent of Japan's population are served by waste-water treatment plants, and many people (63 per cent) do not have flush toilets.

The report points out that since 1970 SO_x emissions have fallen 82 per cent and NO_x 21 per cent, and CO₂ emissions are among the lowest for OECD nations calculated per capita and per unit of gross domestic product. This decoupling of economic growth and emissions of pollutants is held up as a shining example for other nations to follow.

The report explains that it has been achieved through a combination of regulatory measures, by both central and local government, more efficient use of energy by industry and the spread of desulphurization and denitrification technology.

Changes in the structure of the national economy away from heavy polluting industry and greater use of non-fossil fuel energy sources, such as hydropower and nuclear power, have also played an important role.

Water treatment, however, has not been a success. Much raw or poorly treated sewage is dumped into the sea, rivers and lakes, and most sewage treatment plants use primitive activated sludge processes that fail to remove phosphorus and nitrogen compounds.

Indeed, the report illustrates how, despite Japan's 1972 Nature Conservation Law, which states as a "unique cultural tradition of Japan" that "man, nature and man's works of art form an organic unity", there is little sign that this basic philosophy has been translated into action.

Over a 13-year period from 1973, for example, the area of Japan's natural forests decreased by 14 per cent due to development. Most of Japan's lakes have also suffered "severe alteration" during this period and more than 30 per cent of lake shorelines have been developed for industrial or urban use.

Seven hundred kilometres of coastline were modified artificially in the 1980s, and less than half (46 per cent) of the coastline of Japan's four main islands remains in a natural state. More than a fifth (21.5 per cent) of Japan's vertebrate species are either rare, vulnerable, endangered or extinct.

Two million hectares of land has been designated as "national parks" but commercial exploitation (ski tows, spas and golf courses) often occurs on private land within the parks, leading to significant environmental degradation. Japan has one of the lowest percentages (6.4 per cent) of protected land areas among OECD nations. And there is a severe shortage of manpower to look after these protected areas.

The report points out that the various ministries and agencies with responsibility for environmental matters "seem to act in a spirit of competition rather than working together", and it calls for greater cooperation and a comprehensive environmental plan at the national level. **David Swinbanks**

OECD Environmental Performance Reviews — Japan (210 pages, OECD, Paris, 1994).

Gene therapists face double check

Tokyo. University medical researchers in Japan are at last in a position to start preparing for the application of gene therapy techniques to patients, following the setting up of a screening committee by the Ministry of Education, Science and Culture (MESC), which has jurisdiction over universities.

The first meeting of the committee was held in Tokyo last week. Its establishment completes the formation of a government regulatory system for gene therapy in Japan. But, in a typical example of the bureaucratic duplication of effort that occurs in central government, proposals from university researchers will also have to be screened by another similar committee, with overlapping members, established in February by the Ministry of Health and Welfare (MHW) (*Nature* 367, 502; 1994).

The MESC committee is headed by Yoshio Okada, chairman of the Senri Life Science Foundation in Osaka. Among its 16 members are Kenichi Matsubara of Osaka University, Kumao Toyoshima, head of the Center for Adult Disease in Osaka, and Fumimaro Takaku, head of the International Medical Center of Japan in Tokyo, who also serve on the MHW committee.

But there are only three non-scientists on the ministry of education committee, as opposed to five in the MHW committee. This is likely to result in a differing focus for the two committees, and it remains to be seen whether the two committees always agree in their decisions.

Some scientists, including one member of both committees, fear the MHW committee, with its larger complement of non-scientists, may get bogged down in the politics of social and ethical issues. They feel that, as gene therapy techniques will not be applied

to reproductive cells, only the safety and scientific and medical soundness of proposed applications of gene therapy needs to be considered.

But some other key members of the MHW committee consider it important to pay attention to social and ethical issues, and the make-up of the committee, which includes a well-known writer, Ayako Sono, and a former journalist, reflects this view.

In contrast, the MESC committee seems likely to concentrate more on scientific issues. Shin-ichi Ota, of MESC's grant-in-aid planning office, says the main purpose of the MESC committee is to provide expert advice on the safety and soundness of proposals from an "academic" point of view. Social and ethical issues will be debated "where necessary", he says, but coverage of these issues will be "thinner" than in the MHW committee. **David Swinbanks**

Indian medicine to head back to its roots?

New Delhi. Medical research in India is likely to find itself heading in new directions with the appointment of G. V. Satyavati as the first woman to head the Indian Council of Medical Research (ICMR).

Satyavati holds two MD degrees, one in pharmacology and the other in *ayurveda*, a system of ancient wisdom. The combination has provided a basis for her extensive studies of the medicinal values of herbs, and helped her to apply modern tests to traditional cures. Her research, carried out over two decades, led to a herbal drug for treating high blood cholesterol. She was also responsible for manpower development at ICMR,

and handled its publications.

Before her new appointment, she was deputy director-general of ICMR. Observers say her appointment as ICMR chief, where she was chosen in preference to more senior colleagues and others working at the cutting edge of medical technologies, indicates a desire by the government to boost traditional methods of health care.

Satyavati says her immediate priority is to improve medical research in colleges and to revitalize ICMR's many regional units. "We will also take a fresh look at the ethical issues of human and animal experiments," she says. K. S. Jayaraman

Reforms of US energy labs a 'daunting task'

Washington. The task force set up by Hazel O'Leary, the US Secretary of Energy, to advise her on "alternative futures" for the Department of Energy's vast network of laboratories has been warned that efforts at reform face great and perhaps insurmountable difficulties.

The panel has also been told by a former senior scientific adviser to the Carter administration that a crisis of identity in the laboratories reflects a similar crisis facing the department itself, which Carter established in the wake of the Middle East oil crisis in the 1970s.

The task force on the laboratories is chaired by Robert Galvin of the electronics company Motorola, and is due to report next

February. O'Leary sees its report as a pre-emptive strike that could help her to reform the laboratories before Congress looks for more drastic remedies. But with the laboratory network providing high-technology jobs across so many states and congressional districts, her concern may be misplaced.

The network employs 30,000 engineers and scientists and tens of thousands of support staff. It is made up of the three atomic weapons laboratories — Los Alamos and Sandia in New Mexico and Lawrence Livermore, California — as well as a group of smaller but still substantial facilities, such as Fermilab and Argonne in Illinois, and Brookhaven in New York state.

At the panel's first meeting two weeks ago, Galvin says that hearing the experts' warnings was a "valuable process" for the panel, and still believes that the time is ripe for reform of the laboratories. "This is a window of time when it is appropriate for people to listen to us," he says. "We are hoping that because the Cold War is over, budgets are tight, and there's so much interest in industrial competitiveness, people will say that it is time to do something different."

But solutions will be hard to find to the fundamental problem facing the laboratories, namely that government funding for their core tasks of developing both nuclear weapons and energy technologies has been heavily cut back, and replacement options lack credibility.

Lewis Branscomb of Harvard University told the members of the panel that their work had been done many times before, but without any effect: previous studies had been "technocratic analyses that failed to take account of the political context" of the laboratories, he said.

Branscomb said that talk of increased 'technology transfer' out of the laboratories was "the wrong paradigm" for their future. "It implies that there are industrially valuable technologies in the laboratories that need to be pushed [into the marketplace], and that is not the case."

Branscomb expressed concern that moves to save money in the network would have a greater effect on good science laboratories than on the larger but arguably less useful weapons laboratories, as the former had less political clout.

He also came close to calling for the Department of Energy itself to be broken up, arguing that the problem of the laboratories is directly linked to the problems that the department faces. "There is an identity crisis in the labs because there is an identity crisis in the department," Branscomb said. Both seem equally adrift, with no energy crisis to solve or atomic bombs to build.

Congress is also wrestling with the question of a new mandate for the laboratories. It may pass a bill this summer, if the chairman of the House of Representatives Science Committee, George Brown (Democrat, California), can sort out a demarcation dispute with John Dingell (Democrat, Michigan) chairman of the Energy and Commerce Committee and agree on a House version that can be reconciled with a bill already passed by the Senate.

Some voices may demand drastic action. Roscoe Bartlett (Republican, Maryland), for example, is said to be considering moving an amendment on the floor of the House calling for some of the laboratories to be closed down. But even in this cost-cutting Congress, there is no groundswell of support for such a measure. **Colin Macilwain**

German minister takes on Greens

Munich. In a direct challenge to the anti-nuclear policies of the state of Hessen, the German federal environment minister, Klaus Töpfer, has promised to order the state government to grant permission for the completion of a new production plant for mixed oxide (MOX) fuel elements for nuclear reactors.

Construction of the plant, which is located in Hanau and which would have been Germany's only national source of MOX fuel elements, has been suspended at the demand of the state government. But utility companies are doubtful about the impact of Töpfer's challenge, as they feel that he lacks the political stamina for the lengthy battle that would be required to restart the manufacture of fuel elements.

MOX production in Germany, which is controlled by Siemens, has fallen foul of the Social Democrat (SDP)/Green coalition that took control of the government of Hessen in 1991. Both parties are committed to phasing out nuclear power in Germany.

Most of Germany's 20 nuclear reactors now use MOX elements in addition to standard uranium elements. But the original MOX production facility at Hanau was closed down in 1991 by the state environment minister, Josef Fischer, a member of the Green party, after two relatively minor contamination incidents.

Fischer has also continued to oppose the development of the new plant, which would have quadrupled the capacity of MOX fuel produced to more than 100 tonnes per year, and was originally scheduled to start production in 1992.

Siemens had continued to contest the enforced closure of the old plant. Last week, however, it decided to cut its losses and accept that the plant should be closed permanently. Most of the costs of keeping the plant open but unproductive, estimated at more than DM100 million, had been borne by utilities. These have now agreed

that the old plant should be closed, and are negotiating contracts with British Nuclear Fuels (BNFL) in Britain and Cogema in France to produce 300 tonnes of MOX fuel.

Who will produce the remaining 600 tonnes needed to use up the plutonium to be extracted from waste fuel from Germany's reactors depends on the outcome of the battle over the new German MOX plant. Siemens and the utilities have already invested more than DM1 billion in the plant, which is 95 per cent complete and would take around two years to bring into production once construction work started again.

The companies are keen to see a return on their investment as soon as possible. The plant's director, Jürgen Krellmann, claims that permission to complete and operate it is being withheld for purely political reasons. But a spokesperson for the Hessen environment ministry insists that the ministry's sole concern is for the safety of plant workers and the local population.

Töpfer is now trying to break the stalemate. But the situation is not straightforward. Although his federal ministry has ultimate responsibility for nuclear power in Germany, the state government is responsible for safety. He accuses the Hessen government of being *technologiefeindlich* (hostile to technology), but may find it difficult in practice to force his will on Fischer, as he must issue individual orders to approve each technical step in the building programme, and this could amount to 500 separate orders.

Instead, the utilities are hoping for a change in political mood after the October federal elections and next spring's Hessen state elections. But this cannot be guaranteed. The SDP, which is currently leading the opinion polls for the federal elections, is committed to phasing out nuclear power, and promises to ban the production and eventually the use of MOX fuel elements all together.

Alison Abbott

South Africa awaits decision on new science ministry

Cape Town. Now that the African National Congress (ANC) has gained its expected majority in last week's election, the concerns of South Africa's scientific community have become re-focused on whether the government of national unity will include a minister of science and technology.

This decision will be taken shortly by the ANC's national executive committee (NEC). If the outcome is positive, the critical question will be who president-elect Nelson Mandela appoints to the position when he announces the new cabinet at his inauguration in Pretoria next week.

The ANC takes pride in the fact that it is the world's first liberation movement to have drafted a science policy before coming into power. This is partly due to the efforts of NEC member Mohammed Valli Moosa, a strong proponent of the new ministry, whose creation is supported by both the scientific community and the private sector.

As the only scientist near the top of the ANC's list of candidates for the National Assembly, Moosa is also the most likely incumbent. A graduate of the University of Durban-Westville, he taught mathematics and physics for four years before becoming a full-time ANC activist in the early 1980s.

But cabinet positions will be distributed according to the support achieved by the various political parties in the elections. As a result, the prospective science and technology portfolio could alternatively be offered out to either Theo Alant of the National Party, an applied mathematician who is currently deputy minister of finance, or to Zach de Beer of the Democratic Party, a former physician who held a senior position in the Anglo-American Corporation before becoming the party's leader.

One advantage of creating a new ministry is that it will not be required to inherit any civil servants from the old administration, and could therefore provide an avenue for bringing in new talent.

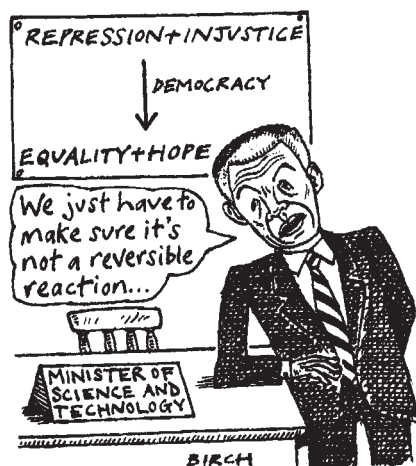
Roger Jardine, science and technology co-ordinator for the ANC, says that what-

ever the outcome of the debate on the new ministry, South Africa will soon have "a much better co-ordinated science system, with clear-cut national goals linked to the reconstruction and development programme".

But whether South Africa ends up with a new ministry for science, or merely a new agency, responsibility for science and technology will be "structurally defined" in the new government, according to Brian Clark, president of the Council for Scientific and Industrial Research.

Rolf Stumpf, president of the Human Sciences Research Council, says he is optimistic about future funding prospects. "If the ANC is at all serious about their reconstruction and development programme, they cannot afford to decrease expenditure on science and technology," he says.

Few South Africans who voted in the country's first democratic election last week were left unmoved by the patient enthusi-



asm of the long queues of people, many of whom were casting their votes for the first time. Science policy was probably not uppermost in most of their minds. But, like many other matters, it has been more hotly debated in the past year than ever before.

Michael Cherry

Chile backs ESO's rights in telescope dispute

Munich. An extraordinary meeting of the European Southern Observatory (ESO) council agreed last week to send a delegation to Chile to discuss with the government the claims by a Chilean family that it is the rightful owner of the 725 square kilometre site for ESO's Very Large Telescope (VLT) at Mount Paranal.

The families' claims have resulted in a prolonged series of court hearings which threaten to prevent ESO from completing

the building of the VLT, planned as the world's largest telescope, in Chile (see *Nature* 368, 676; 1994). ESO was given the land in 1988 by the Chilean government, and wants the government to take a more active role in settling the dispute.

Earlier last week the government moved in this direction, by confirming to the courts that ESO, as an intergovernmental organization, has immunity from local law in Chile.

A. A.

German institute under fire over HIV-blood reports

Munich. The head of the Paul Ehrlich Institute for Sera and Vaccines in Langen, near Frankfurt, became caught up in political cross-fire last week when he was criticized for accepting "six figure sums" from pharmaceutical companies for preparing reports on techniques for inactivating HIV in blood products.

Reinhard Kurth has denied the charges, which he claims are politically motivated, and aimed to discredit the health minister, rather than himself. He is suing his accuser, the Social Democratic Party's health spokesman Horst Schmidbauer, for libel.

Schmidbauer made his accusations shortly before a meeting last week of the parliamentary committee investigating the circumstances surrounding several cases of HIV-contaminated blood products which came to light last October.

Horst Seehofer, Germany's health minister, had responded to the scandal by announcing plans to dissolve the Bundesgesundheitsamt (BGA) — six health institutes directly responsible to the ministry of health through a central administration in Berlin — after complaining that officers had not kept him informed of vital issues.

But Seehofer's opponents are seeking to weaken the political credibility he gained by this action by claiming that he has little effective control over the institutes for which he is responsible.

The Paul Ehrlich Institute, like the BGA, is directly responsible to the ministry of health. In the early 1980s, it was the only institute in Germany with wide experience with retroviruses, and was approached for advice from blood product manufacturers worried about HIV contamination.

Between 1984 and 1985, Kurth and his colleagues — with approval of the ministry of health — prepared reports about the inactivation of the HIV virus in blood products for Behring, Armour Pharma, Immuno and DRK Institute Springe, as well as the Red Cross Blood Bank.

Kurth and Seehofer told the parliamentary committee that DM90,000 had been received for preparing the reports. One-fifth went to the ministry, and the rest was divided between Kurth and his co-workers.

But Schmidbauer claims that more money was involved, and argues that the ministry was wrong to have allowed researchers paid from public funds to carry out work for private companies.

Meanwhile Parliament has approved a new structure proposed by Seehofer for the BGA. This abandons the central administration, which had over 300 employees, and reorganizes the BGA into three institutes.

Alison Abbott

NIH panel rejects Persian Gulf Syndrome

Washington. An advisory panel to the US National Institutes of Health (NIH) recommended after a three-day meeting last week that a health survey be carried out of the nearly 700,000 individuals who served in the Persian Gulf during the war with Iraq at the beginning of 1991.

The panel has also recommended that extensive research be carried out into unexplained symptoms being reported by veterans of the Gulf War. But it has rejected the suggestion that the illnesses represented by these symptoms constitute a single medical syndrome, as some scientists and veterans have argued.

The symptoms reported include fatigue, skin rash, muscle and joint pain, headaches, loss of memory, shortness of breath, gastrointestinal and respiratory symptoms, and extreme sensitivity to commonly occurring chemicals. The panel was set up to examine whether the symptoms were caused by conditions in the Gulf, and to decide if they should be classified as a single Gulf War Syndrome.

Many veterans are now unable to work because of such symptoms, but do not qualify for a disability allowance because their symptoms do not fit any of the diagnostic criteria used by the Department of Veterans Affairs when determining eligibility for disability payments. Advocacy groups for veterans add that there are many men and women still serving in the armed forces who are reluctant to report their symptoms for fear of being labelled as malingerers.

Last week's meeting at the NIH was prompted by a combination of the veterans'

anger and pressure from Congress. At one point Major General Ronald Blanck, commander of the Walter Reed Army Medical Center in Washington, called for the panel to define a Persian Gulf Syndrome. Even if that was not the best scientific opinion, he said, some definition was necessary to enable the veterans to claim disability.

After a private discussion lasting until the early hours of Friday morning, the panel concluded that there is no such thing as Persian Gulf Syndrome. But it acknowledged that people were suffering real illnesses. Herbert Schaumburg, a panel mem-

make its recommendations for extensive research and a health survey.

A further complication is that the actual exposure often differs from the official history. One example concerns pyridostigmine bromide (PB), a drug administered to reduce the effects of nerve gas, which was given to nearly 400,000 troops.

The troops were supposed to take the drug for 72 hours at a time, interrupted by 24 hours, during periods of heightened alert for attack by chemical weapons. In fact, says Irving Cohen, a physician with the Veterans Administration Medical Center in Topeka, Kansas, some units took the drug "every day they were in the Gulf".

PB has unpleasant side effects because it works in the same way as a nerve gas. Both PB and nerve gas bind to and inactivate an enzyme (acetyl cholinesterase) that breaks down acetyl choline — the compound which stimulates nerve action. Nerve gas binds irreversibly, and the resulting lack of enzyme to break down acetyl choline leads to a massive overstimulation of muscle. The body is unable to synthesize new enzyme in time to prevent death.

PB was given to the troops because it binds reversibly to acetyl cholinesterase. It works on the principle that if it is bound to between 20 and 40 per cent of acetyl cholinesterase, the binding can be reversed after a nerve gas attack, freeing up enzymes to break down enough acetyl choline to reduce muscle spasms.

The DoD defends its distribution of PB on the basis that much higher doses of PB have been administered for years to people suffering from myasthenia gravis, with few side-effects. For this reason, the panel accepted that PB was unlikely to be the main cause of some of the symptoms now being experienced. But they did not rule out PB completely, as little is known of how stress affects the body's response to low doses.

Although the NIH panel did not agree on the cause of the range of illnesses being observed, it did conclude that post-traumatic stress syndrome, as well as a new form of the parasitic disease leishmaniasis, could each be attributed to service in the Gulf. So far 31 cases of leishmaniasis have been confirmed among Gulf veterans, but the panel believes there may be more subclinical cases, and a ban therefore remains in force against Gulf War veterans donating blood.

The panel did not rule out any of the suggested reasons for the symptoms being observed, including reports from troops that they were exposed to chemical and biological warfare. Eula Bingham of the University of Cincinnati College of Medicine, said there was no reason to disbelieve a Czech government report that troops had been exposed to chemical weapons. **Helen Gavaghan**

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Did safety precautions cause later illness?

ber who is chairman of the Department of Neurology at the Albert Einstein College of Medicine in New York, said: "I came here with a mind-set that these people were malingering, but I am now convinced that there is real pain and suffering."

Many veterans, who travelled to the NIH campus at their own expense, used the meeting to attack the US government, creating an emotionally-charged atmosphere very different from the usual academic mood of an NIH consensus meeting. By the third day, veterans advocates admitted that the NIH panel was behaving with integrity, and aimed their anger at the Departments of Defense (DoD) and Veterans Affairs.

Some panel members appeared to feel the same frustration with the DoD as did the veterans. When asked if they were satisfied with the DoD's response to the veterans and to their work, most panel members looked uncomfortable, even though remaining silent.

But John Spengler, of the Harvard School of Public Health, complained that, even though it was nearly three years since the end of hostilities, basic information was still not available. "Even when troops were exposed for a long time, we were unable to find any records of exposure."

The Department of Veterans Affairs has information on the health of only about 20,000 of the 700,000 American men and women who served in the Gulf. Furthermore, there is no detailed knowledge about which of the many noxious substances in the Gulf each person was exposed to. It was the lack of such knowledge that led the panel to

Boyer makes gift of \$24 million to UCSF

San Francisco. Herbert Boyer, emeritus professor of biochemistry at University of California-San Francisco and co-founder of Genentech Inc., one of the most successful US biotechnology companies, has donated \$24 million to the school, the university announced last week.

The gift consists of Boyer's portion of the patent rights to the process of splicing genes, estimated to be worth about \$24 million over the patent's 17 years of life up to 1997. Boyer and Stanley Cohen of Stanford University are both credited with the discovery and both universities have shared patent rights with the inventors. It is one of UCSF's most lucrative patents.

The university said it would use the gift to the department of biochemistry to support the development of advanced biomedical research. It is the largest personal donation made to any of the nine University of California campuses. **S. L.**

Humane use of animals

SIR — The selection of Peter Singer as reviewer for *Monkey Business* and *In the Name of Science*¹ serves neither the purpose of objectivity nor intellectual criticism. *Monkey Business* is a partisan account of the explosive events that put People for the Ethical Treatment of Animals (PETA) on the map. As a reviewer of this book, Singer provides no balance: he is of the same philosophical bent as the book's author and is widely recognized as the intellectual father of the current animal rights movement. Although most of your readers are probably aware of the Silver Spring monkeys case, few of them are likely to have more than a sketchy recollection of the hotly contested charges and counter-charges that the case involving Edward Taub has engendered during the past decade. Singer's review adds clarity to neither *Monkey Business* nor the debate.

Singer selectively rehashes a few disputed facts in the case but neglects even to mention the affiliation of the author, Kathy Snow Guillermo, with PETA and glosses over the huge financial benefits PETA has reaped by utilizing the hapless primates as bait for a decade of direct-mail fund-raising. He simply uses the credibility granted to him as a book reviewer for *Nature* to enshrine through repetition a distorted version of the case. He is entitled to his opinions, but *Nature* surely owes its readers a better analysis than he provided.

Singer states that Taub was prosecuted for animal cruelty by the state of Maryland in connection with his alleged treatment of the 17 monkeys under his supervision at the Institute for Behavioral Research. He notes that Taub was convicted of only a single count of the more than a hundred initial charges, and that this guilty finding was set aside later on the "scarcely reassuring ground that Maryland animal protection laws did not apply to experimenters receiving federal grants". The Maryland Court of Appeals may have decided the Taub case on the issue of jurisdiction, but its reasoning vindicated Taub on two grounds: his research (which addressed problems of stroke rehabilitation by creating in monkeys a comparable loss of sensory perception) fell within the state statute's exemption for "normal human activities to which the infliction of pain to an animal is purely incidental and unavoidable" and, as federally funded research with regulated species, it was already subject to multiple federal regulations concerning humane animal care and use.

Singer also misses the mark in his review of *In the Name of Science* when he suggests that the "animal research industry oppose[s] every attempt by the animal rights movement to achieve the most

moderate gains in animal welfare". The undersigned members of the scientific community have engaged in cooperative efforts with many animal welfare groups. The scientific community has sought to improve laboratory animal care. Scientists participated in the development of the US Animal Welfare Act regulations, and we are involved in efforts to ensure adequate funding for the US Department of Agriculture's Animal and Plant Health Inspection Service, which enforces those regulations.

In the United States, the constructive dialogue between the research community and moderate animal welfare groups has produced regulations that ensure that research animals are humanely treated as they contribute to the development of treatments to alleviate suffering of animals and humans alike. But it must at the same time be recognized that it is impossible for researchers to find common ground with the most extreme organizations and individuals, whose goal is the abolition of animal use in research, rather than the improvement of laboratory animal care.

William H. Dantzer

(President, American Physiological Society, 9650 Rockville Pike, Bethesda, Maryland 20814-3991, USA); **Larry R. Squire**

(President, Society for Neuroscience);

Robert G. Petersdorf (President, Association of American Medical Colleges);

Joel G. Hardman (President, American Society for Pharmacology and Experimental Therapeutics); **David Johnson** (Executive Director, Federation of Behavioral, Psychological and Cognitive Sciences, representing 18 national societies).

SIR — Singer¹ perpetuates an extremely misleading view of the legal outcome of the accusations against Taub. And a fact that became available only after the second trial — namely that an acute infection that led to amputation of one monkey's arm was acquired well after the monkey was taken from Taub's laboratory — makes it a virtual certainty that Taub would have been factually acquitted on this final charge if the case had not been dismissed on the basis that Singer noted.

In furthering the appearance that Taub neglected his animals, Singer ignores important evidence that the court found compelling. It was documented, for example, that in the period immediately before the raid on Taub's laboratory, an animal caretaker had been absent from the laboratory on only one day in more than a year. Significantly, an animal caretaker was present at the laboratory every day of Taub's previous vacation. Practices during the period near the time of the raid, however, departed markedly from this

pattern. (Taub was on vacation for the 15 days immediately before the raid.) Two caretakers had been assigned to care for the animals during Taub's absence. The two were to alternate duty each day. During the 15 days before the police raid, which was instigated by PETA, the caretakers were absent a total of seven days. They were absent for three consecutive days before antivivisection visitors were secretly brought to the laboratory by the PETA member who under false pretences had gained a job in the laboratory. They were absent for the three days before and including the day of the police raid, and for one additional day as well.

As the caretakers testified to different excuses for each absence, the probability of the foregoing occurring by chance is less than one in several million. It was such evidence that led to the court's acquittal of Taub.

Referring to Taub's surgery, Singer asserts that "officials at NIH were later to admit that the fractures, dislocations, lacerations, punctures, contusions, abrasions with accompanying infections, acute and chronic inflammation and necrosis are not the inevitable consequences of deafferentation". The assertion to which Singer refers appears to have been made in a published discussion of the case in the *Neuroscience Newsletter* (Fall 1983 Vol. 14 Part 5 p. 7). Singer did not point out that the NIH veterinarians making the assertion had no previous experience in the treatment of deafferented monkeys. He also did not mention that their assertion was vigorously disputed in the newsletter by the neuroscience veterinarians who investigated Taub and who had prior experience in the treatment of such monkeys.

Finally, Singer writes that "perhaps the most disturbing aspect of the case is the way the US scientific community rallied to support Taub. On the evidence produced in court, there can be little doubt that, legal technicalities aside, and whatever one thinks of the nature and value of the research itself, Taub failed to provide basic veterinary care to the monkeys in his laboratory."

His assertion is in striking contrast to the outcomes of the trials and the final opinion of the US Public Health Service Grant Appeals Board. After its investigation, that board did uphold the termination of Taub's grant on technicalities. The board pointedly did not say, however, that any of these technicalities involved harm to the monkeys. This is what the board concluded: "In summary, the record does not support a conclusion that the monkeys actually were harmed by the lack of regular veterinary supervision, or that the condition of the monkeys showed inadequate veterinary care". Why should anyone find it disturbing that, after independently considering the same facts, the

different panels of scientists investigating the case for their professional societies came to the same conclusion as the courts and the US Public Health Service Grant Appeals Board?

Neal Miller

David Johnson

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SIR — In his recent review, Singer¹ inaccurately represents the role of the American Psychological Association (APA) in Taub's defence. APA, along with several other scientific societies, did indeed provide early and crucial support for Taub. However, when Singer's review describes the APA's monetary support of Taub's legal defence, he fails to point out that these funds were allocated only after an extensive investigation of Taub's research by APA's ethics committee that found no ethics violations.

The conclusions reached by the APA ethics committee and similar boards from other scientific societies were the same as those of the court that acquitted Taub of all but one of the charges against him; the same conclusions were reached by the US Public Health Service Grant Appeals Board that reviewed the case on appeal.

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SIR — As the veterinarian mentioned by Singer¹ who testified on Taub's behalf and later "was appointed director of the office of Animal Research Issues at the National Institute of Mental Health", I would like to comment on his inaccuracies and apparent lack of medical knowledge (also evidenced in his book, *Animal Liberation*² as Russell and Nicoll³ have revealed).

Had I not earlier helped to treat monkeys with removal of sensory inflow to the limb (deafferentation) and known that, short of amputation, only palliative, nursing care was possible, I would not have testified. Despite the ugly appearance of the limbs, due to their being treated as foreign objects by the monkeys and the effects of disturbed circulatory reflexes — not lack of care — the monkeys were in otherwise good health: alert, well-fleshed and with gleaming hair coats. Comments about the sequelae of deafferentation by "NIH officials" were ill-informed, as I stated in 1983 in response to that statement. Taub's veterinary consultants in the past — Singer leads one to believe Taub had never consulted veterinarians — had reached conclusions similar to mine and those of my veterinary colleague, Peter

Hand.

Ironically, veterinary care at NIH by those new to the problems presented by the chronically deafferented limb appears to have led to the amputation of the arm of one monkey (Nero) to counteract gangrene. Another animal, Paul, almost lost an arm for the same reason. This was apparently induced by bandaging the arm despite Taub's warning that circulation could become compromised due to swelling. But it was Taub who was convicted by a non-medically trained jury of inadequate veterinary care of that monkey alone (Taub's conviction on that one issue was reversed by the Supreme Court of Maryland). Neither the prosecutor nor NIH ever showed him the pathology report on that limb, which stated that the osteomyelitis his allegedly chronically poor care induced did not exist. Rather, there was osteonecrosis from a compromised blood supply with evidence of bandaging.

Adrian R. Morrison

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1. *Nature* **367**, 523; (1994).
2. Singer, P. *Animal Liberation* 2nd edn. (Random House, 1990).
3. Russell, S. & Nicoll, C. *Proc. Soc. exp. Biol. Med.* (in the press).

SIR — The leading article "Animals in research" (*Nature* **368**, 84; 1994) incorrectly asserts that "the most adamant . . . defenders of animal research show no signs of wanting to compromise" on the use of animals in research. Thus medical researchers are mischaracterized as counter-extremists to the animal rights movement. That is to suggest that medical researchers believe that humans have an unrestricted right to use animals in any way they choose. This is far from the medical community's position.

Medical researchers are in agreement with the vast majority of Americans on the issue of animal research. They believe that it is appropriate to work with animals in the pursuit of cures and treatments as long as the animals are treated as humanely as possible. This concept is known as animal welfare. Not only is it widely accepted by the medical research community, and has been for years, but it is also enforced by strict laws and regulations (medical research is among the most regulated activities in the country).

What do animal rights leaders say about the "middle ground" concept of animal welfare? Animal rights philosopher Tom Regan said, " . . . the enactment of animal welfare measures actually impedes the achievement of animal rights". Thus they consider their extreme position to be not only correct, but also necessary to

reaching their goal.

According to Dr Adrian Morrison, a representative from the National Institutes of Mental Health: "In order to have a rational discussion regarding animal research, the discussion must be amongst those who recognize it's appropriate to work humanely with animals."

Therefore, unless animal right extremists abandon their radical philosophy and join the majority of Americans — including medical researchers — who believe fervently in the humane use of animals, a rational discussion cannot occur.

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Nature/nurture

SIR — The persistent controversy about the nature/nurture dichotomy, as recently reanimated by John Maddox (*Nature* **366**, 107; 1993) and commentators (**367**, 591; 1993), can be resolved most easily if the interaction between organism and environment itself is integrated into a broader comparative approach. In ethological research for example, this is nothing more than the standard method of establishing phylogenetic trees of behaviour patterns. In such a perspective, it is quite trivial to demonstrate environmental influences on genetic factors as there exists not a single gene which, through its phenotypic effects (behaviour included), could be proved to be absolutely independent of the milieu. It is, however, not trivial to examine how genetically different systems react to exactly one and the same external influence. Given the fact that the interpretation of concrete 'stimuli' by a living system typically depends on the specific genetic structure of the organism involved in the interaction, the Strong Genetic Principle is indeed valid as the universal information processing factor in evolution. This does not at all mean that genes are independent of their environment and thus would predetermine even the fate of human beings. Rather, it is the characteristic way in which they interpret influences from an ever changing milieu which is absolutely independent of any external instruction.

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Letters submitted for Correspondence should be typed, double-spaced, on one side of the paper only.

In search of European excellence

F. Gros and G. P. Tocchini-Valentini

What should be the priorities for supporting life-science research in Europe? This article, an upshot of an initiative by Commissioner Ruberti of the European Union, addresses the question.

THE new Framework Programme of the European Union, which will provide funds for scientific research and technological development from 1994 to 1998, is the subject of intense debate. The programme is proposed by the European Commission but then has to be decided upon jointly by the Council, made up of the science ministers of the member states, and the European Parliament. The result will be the definition of the scientific and technological objectives of the Union for the next five years. Support will be provided for research, technological development and demonstration programmes; for scientific cooperation with countries outside Europe and international organizations; for programmes to encourage the application of the results of research to practical ends; and for encouraging the training of scientists and their mobility within Europe.

Whatever the point of view from which one examines the specific programmes within the Framework, the long-term aims, for example the improvement of industrial competitiveness in Europe, cannot be achieved if the scientific work is not evaluated and supported according to the criterion of excellence. Commissioner Antonio Ruberti of the European Union, who has responsibility for science, research and development, has been keen to solicit independent scientific advice. To this end, he has appointed advisors to provide him with their views on research trends in the life sciences (F. Gros), high-energy physics (C. Rubbia) and applied mathematics and complex systems (I. Prigogine). As part of this process we chaired a meeting of 25 scientists with expertise in biological, biomedical and biotechnological research in Brussels at the end of October 1993. The object was to arrive at specific recommendations that might form part of the basis for the Commission's deliberations. This article is the summary of our discussions*.

The science

The following areas of research particularly require action by concerted means.

We believe that they meet the criteria of the fourth Framework, in that they will serve to increase the competitiveness of European industry, and will also contribute directly to improvements in the health of European societies.

1. Structural biology. One of the main problems in modern life-science research is that we do not know enough about the relationship between primary structure, (nucleic acid or protein sequence), and the tertiary structures of biologically active macromolecules, and we know even less about the quaternary structures of the multi-subunit complexes that mediate most biological activities. So we cannot yet accurately predict biological function from structural motifs.

Advances in biological structure determination mean that data collection is now relatively rapid, and that it is possible to work out the structures of protein-protein complexes and of complexes between proteins and other macromolecules (DNA, RNA, carbohydrates and lipids). Understanding the structural basis of such complex formation is essential to the discovery and refinement of new chemical entities which either modulate or interfere with complex formation, and is thus of direct relevance to the pharmaceutical industry. Studies of RNA, which can act as both a genetic message and an enzyme, will be of importance and interest, particularly given the role of RNA in the evolution of life.

This research will require the continued application of X-ray crystallography, nuclear magnetic resonance spectroscopy, electron microscopy and confocal microscopy, and work to improve the resolution of these techniques and the size of structure that they can analyse will be crucial. These technical developments will allow more detailed studies of sub-cellular structures — chromosomes, spliceosomes and replisomes, for example — with further implications for both biological understanding and health-care provision.

2. Developmental biology. Developmental biology deals with the mechanisms by which the three-dimensional structure of the organism and its many and complex functions are derived from the fertilized egg. Central to understanding this process is knowledge of how the body plan of the embryo is first established, how the information in that body plan is subsequently interpreted, and how morphogenetic processes (for example cell-cell inter-

actions and cell migration) give rise to the final organism. It will also be of great importance to study the development of particular structures, for example the brain. Studies of the regulation of gene activity in both time and space are relevant to all of these issues. Our view is that research in this area under the Framework Programme should be confined to a few experimentally tractable model organisms.

Understanding how organisms are constructed, how cells are instructed as to what they should be, and where they should be, is essential to finding out how these processes malfunction in disease. Such knowledge is also highly relevant to research into regenerative processes in adult organisms, for example wound healing and the repair of the nervous system. Work of this sort is particularly timely because of the advent of technologies for manipulating the genome of intact vertebrates and because of the availability of techniques that enable us to identify the molecular signals that induce cellular responses. Of particular interest will be the identification of such triggering molecules, the receptors with which they interact, and the internal cascades of responses that ultimately regulate the activities of transcription factors and lead to adaptive patterns of gene expression. These events are central to the modulation of differentiation and in determining normal as well as abnormal development. The new technologies involved represent the biological revolution of the 1990s, which will be equivalent in its effects to the revolution of the 1980s based on recombinant DNA technology.

These advances will allow us both to model and to understand complex disease pathologies. They are thus of direct relevance to health care and to the needs of the pharmaceutical industry, as they will lead to the availability of test-beds for the development of new drugs and gene therapies.

3. Genome research. The study of the human genome is central to improved understanding of the genetic basis of inherited diseases such as cystic fibrosis and thalassaemia, as well as complex clinical conditions such as cancer, heart disease and Alzheimer's disease. Structural analysis of genomes must go hand in hand with studies of the mechanisms of gene function. In this regard, in addition to the human genome it will be necessary to

*This report appears under the names of the co-chairmen of the Brussels meeting, F. Gros and G. P. Tocchini-Valentini, but was contributed to by all participants. They were: M. Bishop, M. Buckingham, A. Capron, L. L. Cavalli-Sforza, S. Cusak, A. Falaschi, R. B. Flavell, A. Garcia-Bellido, M. Grunberg-Manago, P. Gruss, K. Hoffman, R. Huber, M. Kafatos, U. Laemmli, G. Nicolis, R. Rajewsky, P. Rigby, J. Schell, P. Slonimski, D. Von Wettstein, J. Weissenbach, R. Williamson and A. Xavier.

concentrate on a few carefully chosen model systems (*Bacillus subtilis*, yeast, *Arabidopsis*, *Drosophila* and the mouse). The mouse is of crucial importance in that, at the moment, it is the only practicable model for human disease pathology. Our view is that comparative genome mapping has a central role in maximizing the efficiency with which the results of genome analyses are converted into biologically useful information.

4. Health and agricultural research. In the health sector, newly identified molecules and cellular processes will surely become essential tools, so long as they can be fully developed in the context of the physiology of animal and human diseases. The design and use of animal models for gene targeting (so-called 'gene knockout') experiments is now indispensable to strategies for disease control. Moreover, the development of new vectors will be essential for most future applications of somatic gene therapy, a potential route for treating inherited diseases, cancer and possibly AIDS. Particular attention should be given to work on infectious diseases, especially vaccine development where more concerted European efforts are needed. Other notable areas that we should continue to address are the basic biology of cancer, the pathogenesis of autoimmune diseases, the study of the brain in health and disease, and the development of gene therapy.

Both agriculture and our environment will benefit from the possibility that we will have a complete molecular understanding of plant biology. Of particular priority will be studies of the physiological responses to stress and pathogenesis, the metabolism of the storage products that make up the chemical diversity of the biomass, and the modulation of heterologous gene expression. The introduction of specially designed genes will pave the way for the efficient use of plant processes for the production of specified end products.

A selective programme of plant science research, bringing together those who create the technology and those who need to use it, would be of great value in addressing agricultural, industrial and environmental issues within the Union. Much of this research will be applied to creating environmentally sustainable crop systems and new options for integrated production and processing chains. New substrates for the food industry, improved processes, and assays for determining quality and quantity of food attributes will become available.

The infrastructures

The efficient pursuit of life-science research in Europe depends upon the provision of certain infrastructures that are beyond the means and competence of national authorities and private initiative. These facilities must be responsive to user

needs, and there must be mechanisms for ensuring their quality in that respect.

1. Genetic archives and stock centres. The ability to subject any gene to specific mutation opens the way to a revolution in our understanding of the normal function of genes and their malfunction in pathological processes. But it is beyond the means of even the best funded national laboratories to maintain the stocks of all of the mutant animals that they will generate. Moreover, much of the benefit of this technology will come from the interbreeding of such mutants, to create organisms with multiple defects. It will also be essential for researchers to be able to re-access mutants that were made for a particular purpose, for use in research that could not have been predicted at the time that the mutant was first made. So we need to ensure that all such mutants are freely available to researchers within the Union and that the information available to each generation of researchers is not lost.

We therefore propose that the community should support the best initiatives aiming to establish European repositories for three model organisms relevant to modern genetics, biomedicine and agriculture.

A repository of mouse (and rat) mutants will be indispensable for work on gene interactions, which is itself vital for studies of the diseases (such as cancer and coronary heart disease) that are the main causes of illness and death in the Union. The activities of this repository should, to some extent, be modelled on and be complementary to those of the Jackson Laboratory in Maine in the United States. Likewise, a European stock of *Drosophila* mutants is desirable, because this organism has and will be the source of a great deal of information on gene structure and function, as are European plant repositories (for reasons that are identical in principle but different in details).

2. Bioinformatics. The provision of central facilities for biological information storage, retrieval and analysis also transcends national boundaries. The availability of up-to-date and professionally curated data libraries, as exemplified by the data library at the European Molecular Biology Laboratory, is essential for all of European biology; the accumulation of genomic sequences and protein-structure information will deepen this need. Core European databases would have to be developed in such a way as to prevent duplication of effort and waste of funds. The Union should assure, and possibly expand, the provision of such strategic services, providing the necessary funds to the centre or centres that creates and maintains high standards and user satisfaction. Furthermore, there is a clear need for a European database of protein structure and function. Union funds should

support the development and integration of such core services with other, higher order, specialized databases, thus ensuring coordination and the broad distribution of all sources of biological data through the network of national nodes for bioinformatic users.

The efficiency of European endeavours in this area will depend on two features, centralization and synergy. On the one hand, the existence of single entry points for each service will save resources and simplify the submission of information and subsequent access to it. On the other, sharing resources through a network in which the minimum number of data providers serves the largest community of users will have synergistic effects.

3. Training and mobility. Schemes for training researchers and encouraging the mobility of scientists within the Union are of great value. In formulating action to be taken under the new Framework Programme, it will be necessary to consider past experience and address new needs. The trans-national twinning or networking of laboratories collaborating on research is crucial and should receive additional support. We should, we believe, return to the flexibility of the SCIENCE programme. In particular, there should not be quotas for different budget items — the types of personnel required and details of budget allocation will depend on the nature of the project.

Fellowships for training through research will have to take into account current worldwide trends, for example the lengthening of the post-doctoral period, which in the life sciences is now 3–4 years. Such fellowship programmes should also provide adequate research support to the host laboratory and be set up so as to encourage training in an interdisciplinary setting. We strongly support the idea that the European Union should begin to fund formal courses to prepare young scientists for the use of advanced facilities.

Now that there is a healthy level of mobility of post-doctoral trainees within Europe, the similar needs of young, independent researchers ought to be addressed. We would favour a programme to support excellent young scientists (for, say, seven years) in establishing a research group outside their country of origin. Such an initiative would help create a truly European scientific community and would contribute to the independence of scientists during their most creative years; moreover, by acknowledging the special needs of less favoured regions, it would strengthen the overall cohesion of the European community. □

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Hubert Humphrey's bladder cancer

PCR techniques, retrospectively applied, reveals bladder carcinoma nine years before it was clinically apparent and provides a dramatic focus for the debate about the use of molecular techniques in the diagnosis of cancer.

HUBERT Horatio Humphrey, the vice-president of the United States from 1963 to 1969, died of bladder cancer on 13 January 1978. A diagnosis of infiltrating carcinoma of the bladder had been made in 1976, following the identification of a "borderline malignancy" in 1973. But the first signs of Humphrey's bladder cancer were present nine years before his death. Humphrey first went to his physician with symptomatic blood in his urine in 1967. Visual cystoscopy of the bladder revealed what looked like chronic proliferative cystitis, but an examination of bladder tissue obtained by biopsy gave only inconclusive results.

Pathology remains part art, part science. In 1967 that was surely so. A number of pathologists independently reviewed Humphrey's case: one of them, John K. Frost of The Johns Hopkins Hospital, said the vice-president's cells showed signs of malignancy, but several others disagreed and Humphrey's physician quite reasonably adopted a watch and wait strategy.

Who was right? A new generation of Hopkins researchers (with the permission of Humphrey's widow) has taken tissue frozen since 1967 and analysed it using the PCR (polymerase chain reaction) technique. David Sidransky and his colleagues have identified telltale mutations in the p53 tumour-suppressor gene of Humphrey's tissue. According to an article in the 5 May issue of *The New England Journal of Medicine*, a number of cells in Humphrey's urine in May 1967 harboured the same mutation of p53 (on chromosome 17) as one that was also detected in residual samples of the primary bladder carcinoma, surgically removed in 1976.

The mutation appears to be quite specific. Exons 5 to 9 of the p53 gene in DNA taken from primary tumour tissue were amplified by PCR, whereupon nucleotide sequencing revealed a simple "T:A to A:T" transversion in codon 227 of the gene. In other words, the nucleotides adenine and thymine had been interchanged between the opposing strands of the DNA at a specific site. But this mutation was not present in non-malignant tissue from the bladder at the time of the operation.

As far as is known, there is nothing to suggest that Humphrey's bladder tumour was a hereditary form of disease. Indeed, the recognition that tumour cells carry a mutation of p53, and that normal cells do not, is a further vivid illustration of how tumour cells are often distinguished from others by a mutation of some gene that has presum-

ably arisen in the course of the mitotic division of previously normal tissue. In Humphrey's case, what PCR has shown is that the condition of which he complained in 1967 was medically significant, even though the disease did not become clinically apparent for nine years.

It is tempting, in a case as prominent as this, to ask whether, if Frost's diagnosis had been accepted and aggressive treatment begun in 1967, Humphrey might have lived to be president of the United States, thus changing the course of history. It is possible, that had the diagnosis been clear earlier, he would have withdrawn from the 1968 presidential race against Richard Nixon, recently deceased.

But that, of course, is not the point. Rather, the case of Humphrey's bladder cancer is intended vividly to show that the PCR technique is not simply a laboratory tool, but that it is applicable to real medical problems, here and now. Three years ago (*Science* 252, 706; 1991), Sidransky and colleagues from Vogelstein's laboratory at Johns Hopkins described mutations of the p53 gene in bladder cancers and associated urine samples, speculating that detection of such mutations could be important not only for diagnosis and patient monitoring but also in understanding the pathogenesis of the disease.

The data tend to confirm the view that an alteration (which may often be a deletion) in a tumour-suppressor gene is a molecular event that may turn normal cells into proliferating tumour cells. More recent data from Sidransky, Vogelstein and others confirms the association of alterations in the p53 suppressor gene with other cancers, including lung, colon and pancreatic tumours as well as tumours of the head and neck.

This is no small matter. In 1993, approximately 52,000 people in the United States alone were diagnosed with bladder cancer. Many of them will have invasive disease that will not be curable with standard therapy such as surgery or even surgery and chemotherapy. Thus, detecting these tumours early, before they become invasive, can significantly improve survival.

The implications for early diagnosis are obvious: PCR provides a means by which early indications of malignant disease which can be assessed only ambiguously at present may be firmly put on one side of the fence or the other. It could become routine tomorrow. But several practical issues require resolution.

One is the matter of cost when health-care reform and cost-savings are at the top of

the medical agenda in the United States and most other Western countries. Sidransky, whose work is partly supported by ONCOR Inc. (Gaithersburg, Maryland) estimates the cost of PCR analysis of urine specimens at \$25 a test. In the long-run, that would be as nothing if it permitted diagnosis sufficiently early to result in definitive cure of disease.

But is the PCR technique precise enough, and to what range of tumours could it be applied? As with pathology (remember, different pathologists read Humphrey's 1967 slides differently) there is still a measure of art in the application of PCR to human tissue. Mutations of p53 may occur at any number of locations; they are not limited to a single codon even in bladder cancer. Worse yet, while 60 per cent of bladder carcinomas do show p53 mutations under careful analysis, 40 per cent do not. It is also now known that mutations of another tumour suppressor gene (on chromosome 9) are linked with some bladder carcinomas. More will have to be learned of the natural history of mutations in bladder cancer before diagnosis can be certain.

Other applications in the pipeline also offer exciting clinical possibilities. When removing tumours, surgeons usually take out a margin of surrounding tissue, hoping that the pathologists will find "clean" or disease-free tissue. At the recent meeting of the American Association for Cancer Research in San Francisco, Sidransky reported successful molecular diagnosis of residual infiltrating tumour cells left behind in what appear to be pathologically clean margins and lymph nodes of patients with squamous cell carcinoma of the head and neck. "It looks as if even a few residual cells are prognostic of recurrent disease," Sidransky says of the preliminary work.

Clearly this technology needs to be refined and made available at reasonable cost to appropriately defined populations.

Therein lies one of the most important challenges in biomedical science. "We have in our hands an extraordinarily powerful new technique," says Sidransky, "but we have not perfected ways to use it. People out there are dying who could be saved. Hubert Humphrey was only one of them."

The Humphrey story and the issue of developing molecular techniques such as PCR into everyday medical tools illustrates perfectly the challenge that is so often described as "technology transfer" or taking science from the laboratory to the bedside — which is, after all, what biomedical research is really all about.

Barbara J. Culliton

Bad blood by mutation

Philip W. Majerus

VENOUS thrombosis is a serious health problem. It afflicts about 1 in 1,000 people each year, the main disorders being thrombophlebitis of the legs and pulmonary embolism, which result in considerable suffering (and death). The disease is familial in about half of cases, but previously defined single-gene mutations have been linked to thrombosis in only a few of the cases. Now, on page 64 of this

lar blood to clot while maintaining intravascular fluidity. Obviously, however, defects in the pathway could provoke thrombosis, and indeed protein C and protein S deficiency are associated with an increased risk (5–10-fold) in heterozygotes. To date, prothrombotic mutations have not been found in thrombomodulin or thrombin.

The other previously defined gene de-

showed that this test detects an autosomal dominant trait associated with thrombosis, a finding confirmed by Bertina and co-workers⁴ and Griffin *et al.*⁵.

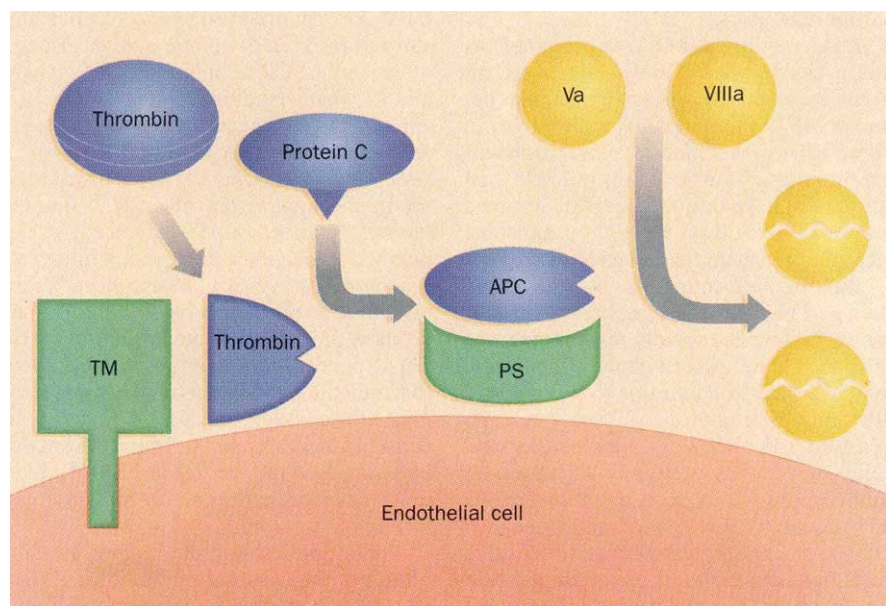
Dahlbäck has purified a factor from human plasma that corrects the defect, and he showed that it is coagulation factor V (ref. 6). In the latest paper¹, Bertina's group has defined the molecular basis for the assay phenotype. Patients that have resistance to APC have a single mutation G→A that converts arginine 506 of factor V to glutamine. This mutation is at the site for cleavage by APC. Cleavage leads to inactivation of factor Va, which nicely explains the thrombotic risk because these factor V molecules are resistant to APC but have normal factor V procoagulant activity.

The importance of these findings is that the mutation concerned is very common. At the second Pine Ridge conference on the genetic causes of thrombosis held at Snowbird, Utah, in March of this year, Bertina estimated that 2–4% of the Dutch population carry the mutant allele and Dahlbäck estimated that 7% of the Swedish population has the defect⁶. It was also reported that the same mutation is present at similar prevalence in the United States and in various ethnic groups worldwide.

As with the previous mutations associated with thrombosis, the factor V defect is a risk factor in which only a small fraction of affected individuals have disease. The mutation cropped up in 56 out of 301 of a consecutive series of patients with venous thrombosis¹, and in 40% of patients referred to a centre for coagulation disorders⁷. Thus the mutation — and therefore the risk — is about 5–10 times the general population frequency. Homozygotes for the mutation may be at greater risk than heterozygotes; 6 out of the 301 patients were homozygotic for it, which is about 25 times more frequent than predicted from the population data. In families with thrombosis the effect of the mutation seems to be much greater, with some 20% of affected individuals having a thrombosis by the age of 50 (ref. 7). This suggests that thrombosis-prone families are afflicted with several interacting gene defects that put them at greater risk. At Snowbird, Bertina confirmed this impression by noting that individuals in thrombosis-prone families with mutations in both protein C and factor V were much more likely to have thromboses (71%) than those with either of the single mutations (13–35%).

The high frequency of a single factor V mutation in diverse groups of people raises the question as to whether positive selection pressure is involved in maintaining it in the population. Perhaps a slight thrombotic tendency confers some advantage in fetal implantation.

Bertina and Dahlbäck have studied



The protein C anticoagulant pathway. Procoagulant thrombin binds to endothelial thrombomodulin (TM), converting thrombin to an anticoagulant that activates protein C to activated protein C (APC). With its cofactor protein S (PS), APC inactivates coagulation factors Va and VIIIa.

issue¹, Bertina and co-workers report that a missense mutation in the coagulation factor V gene is found in about 50% of familial thromboses.

Procoagulant and anticoagulant forces are delicately balanced in humans by a complex system of cofactors and inhibitors. Over the past ten years the role of the thrombomodulin/protein C anticoagulant pathway has been defined². This is a feedback system in which all of the components are derived from elements in blood and several are products of the coagulation process itself (see figure). As thrombin is generated at sites of vascular injury, it activates and aggregates platelets and clots fibrinogen. It also binds to the endothelial membrane protein thrombomodulin, this event converting thrombin from a procoagulant to an anticoagulant protease that activates a plasma protease zymogen protein C. The activated protein C (APC) cleaves and inactivates the coagulation factors Va and VIIIa in the presence of a cofactor protein designated protein S.

This elegant system allows extravascu-

lar blood to clot while maintaining intravascular fluidity. Obviously, however, defects in the pathway could provoke thrombosis, and indeed protein C and protein S deficiency are associated with an increased risk (5–10-fold) in heterozygotes. To date, prothrombotic mutations have not been found in thrombomodulin or thrombin.

The situation changed last year with the discovery by Dahlbäck *et al.* of a new assay for defects in the action of APC (ref. 3). Dahlbäck postulated that a defect in the protein C pathway interferes with the anticoagulant action of APC. He devised a simple yet ingenious assay to test this possibility, in which the clotting time of blood is measured in the presence and absence of exogenous APC. In the normal response, the clotting time is prolonged in the presence of APC because of the inactivation of factors Va and VIIIa. A defect is detected as a failure of prolongation of the clotting time resulting from resistance to added APC. Dahlbäck

almost 100 families with thrombosis not associated with any of the previously reported causes outlined above and find that 40–50% show APC resistance. So there may well be other as yet undefined genes that predispose to thrombosis in the remaining families. Moreover, it seems likely that there are other defects in factor V. When activated to factor Va the protein functions as a procoagulant cofactor in activation of prothrombin to thrombin. The anticoagulant actions of V and Va have been subject to much less attention. At least two such functions have been proposed. Thus factor Va but not V stimulates the activation of protein C by thrombin⁸, and factor V but not Va is a cofactor for the action of APC according to Dahlbäck. The sites in factors V and Va that mediate these effects should be investigated for mutations in thrombosis-prone families. Another strategy to find mutations is afforded by the availability of large numbers of patients and families with the common factor V mutation; general genetic linkage analysis of such families may reveal other genetic defects.

What can be done about these mutations? They are a major risk factor in thromboembolic disease, yet most people who harbour the mutant proteins will never suffer a thrombosis. Thus the risks of lifelong treatment with anticoagulants must be weighed against the benefit of preventing infrequent but potentially devastating thrombotic attacks. We need better methods to define those who warrant preventive anticoagulation therapy. □

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1. Bertina, R. M. *et al.* *Nature* **369**, 64–67 (1994).
2. Dittman, W. A. & Majerus, P. W. *Blood* **74**, 1–7 (1990).
3. Dahlbäck, B., Carlsson, M. & Svensson, P. J. *Proc. natn. Acad. Sci. U.S.A.* **90**, 1004–1008 (1993).
4. Koster, T. *et al.* *Lancet* **342**, 1503–1506 (1993).
5. Griffin, J. H. *et al.* *Blood* **82**, 1989–1993 (1993).
6. Dahlbäck, B. & Hildebrand, B. *Proc. natn. Acad. Sci. U.S.A.* **91**, 1396–1400 (1994).
7. Svensson, P. J. & Dahlbäck, B. *New Engl. J. Med.* **300**, 517–522 (1993).
8. Salem, H., Broze, G. J., Miletich, J. P. & Majerus, P. W. *Proc. natn. Acad. Sci. U.S.A.* **80**, 1584–1588 (1983).

TECHNOLOGY

A microstamp of approval

Charles M. Knobler

THE finely patterned surfaces that are essential to the fabrication of microelectronic devices and sensors are generally prepared by 'high-tech' methods such as electron beam lithography. A remarkable lower-tech procedure, closely akin to rubber stamping, was described by George M. Whitesides (Harvard University) at a meeting of the American Chemical Society in March*. This method for preparing patterned surfaces can also be used to produce small three-dimensional structures such as microlenses. The key is the control of surface reactivity and wettability by self-assembled monolayers.

Self-assembled monolayers, or SAMs, form when the sulphhydryl groups of long-chain alkanethiols ($\text{HS}(\text{CH}_2)_n\text{R}$ with n of the order of 15) react with gold surfaces. The reaction occurs rapidly at room temperature and leads to regular, close-packed monolayers, much like compressed films of insoluble fatty acids on the surface of water. Whitesides and his collaborators had already shown that one could draw fine lines of monolayers on gold surfaces by using thiols as the ink (G. P. López, H. A. Biebuyck, C. D. Frisbie and G. M. Whitesides *Science* **260**, 647–649; 1993). The lines are not detectable by optical microscopy, but they can be seen when water vapour condenses on the surface.

Near the turn of the century, it was observed that if one 'wrote' on glass with the tip of a hot flame, the pattern traced by the flame became apparent as a dark line when one breathed on the surface (Lord Rayleigh *Nature* **86**, 416; 1911, and J. Aitken *Nature* **86**, 516; 1911). The patterns, which were named 'Breath Figures', become visible because of differences in wettability. If glass is scrupulously clean, water condenses on it as a continuous film. But when there are minute traces of oily impurities on the surface (even a single monolayer) the water condenses in the form of droplets that scatter light and appear white. The lines can be observed because droplets do not form where the flame has cleaned the surface. Whitesides has coined the less charming but more general term 'condensation figures' for these patterns.

The condensation experiments demonstrated that very fine patterns can be produced with SAMs. The more recent experiments by Whitesides and colleagues have shown that, like ink, the thiols can be applied to surfaces with a 'rubber' stamp, a process that he has named 'microcontact printing'. In fact, the stamps are not rubber; they are made by polymerizing PDMS (polydimethylsiloxane) on a master patterned surface that has been prepared by conventional techniques (see top of Fig. 1).

The pattern is then transferred to a thin

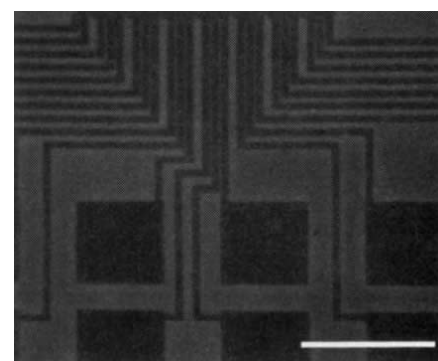
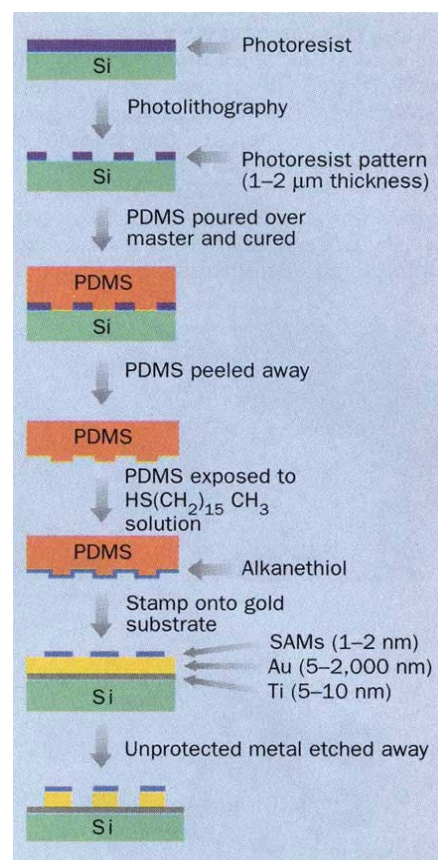


FIG. 1 Top, the preparation of patterned surfaces by 'rubber stamping' and chemical etching. (Adapted from a figure supplied by J. L. Wilbur, A. Kumar, E. Kim and G. M. Whitesides.) Bottom, a scanning electron micrograph of a structure formed by this process. Scale bar, 10 μm . (Photo supplied by A. Kumar, H. A. Biebuyck and G. M. Whitesides.)

layer of gold on a silicon wafer by dipping the stamp in a thiol and touching it to the surface. The liquid deposited by the stamp reacts with the gold in about a second and forms a robust SAM, 1–2 nm thick. Patterns with dimensions from 1 to 100 μm are reproduced faithfully (as shown in the micrograph in Fig. 1), features as small as 0.2 μm less reliably. The same stamp has been used up to 100 times over a period of several months without noticeable deterioration in performance.

The parts of the surface covered by the SAM are relatively inert chemically. Bare

*Spring Meeting of the American Chemical Society, San Diego, California, 13–17 March 1994.

gold surface can be dissolved away by an aqueous cyanide solution, leaving the SAM-covered pattern intact. The pattern also survives subsequent chemical etching of the silicon, although it can be removed by washing with aqua regia.

At the meeting, Whitesides also described how condensation figures on patterned surfaces can be used to create complex three-dimensional arrays of

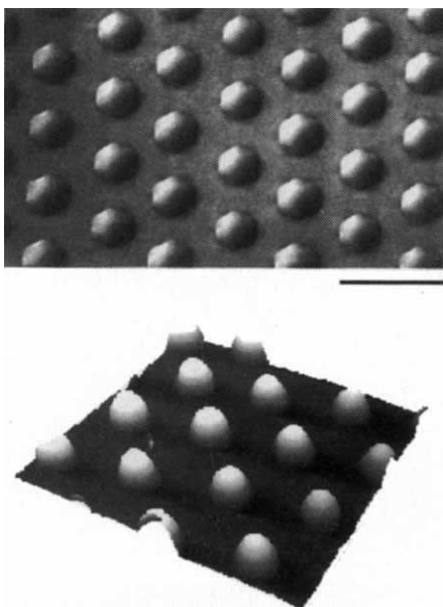


FIG. 2 Atomic force microscopy image of a polyurethane structure fabricated on a patterned surface. Scale bar, 20 μm . (Photo supplied by H. A. Biebuyck and G. M. Whitesides.)

organic materials — for example, tiny polymer lenses. An intricately patterned SAM is stamped on a gold surface and then a second monolayer is formed on the uncovered surface by dipping the wafer into a solution of a different thiol. The wafer is then passed through a thin layer of organic liquid on a water surface. The shape of droplets that adhere to the surface of the wafer is controlled by the SAM pattern and the surface tensions between the two SAMs and the organic liquid. Stable arrays can be produced (Fig. 2) if the organic liquid can later be polymerized, either by exposure to ultraviolet light or by heat.

At the moment, the densities of defects in the final structures produced by micro-printing are large compared with those prepared by conventional means. But the simplicity of the method, and the fact that it can be carried out under ordinary chemical laboratory conditions, make it an attractive possibility for further development. $\square$

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The hole truth about perforin

William R. Clark

SINCE the discovery that certain subsets of lymphocytes can kill other cells in the body, identifying the lytic mechanism used by these killer cells has become one of the holy grails of immunology. It has been no easy task, either for those within the field, or especially perhaps for those trying to follow events from the sidelines. On page 31 of this issue¹, Kägi *et al.* take a giant step towards clearing up one of the most puzzling aspects of this saga — the role played in cell-mediated cytotoxicity by the pore-forming protein known as perforin.

The killer cells concerned include cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. Cytotoxic T lymphocytes are important in the elimination of virally infected cells and the suppression of oncologically transformed cells, and they represent (entirely coincidentally, from nature's point of view) a major immunological barrier to organ transplantation. Natural killer cells are essential for effective tumour surveillance and destruction, and are also problematic in bone marrow transplants. So a precise knowledge of how these cells function is of considerable practical as well as theoretical interest.

The discovery of perforin (also referred to as cytolyisin or pore-forming protein), a complement-like protein stored in cytoplasmic granules in many NK cell and CTL lines, seemed to have ended the search for a lytic mechanism when it was first reported over ten years ago². When CTLs bind to cells recognized as being aberrant or foreign, the contents of these granules (which contain an assortment of enzymes as well as perforin) are released vectorially onto the 'target' cell. Perforin monomers have been proposed to assemble into polymeric pore structures, which insert into target cell membranes and create holes that lead to a rapid cell death bearing many of the hallmarks of programmed cell death, or apoptosis.

The simplicity of the perforin model for cell-mediated cytotoxicity, and its intuitiveness (based on its analogy to complement), led to its rapid and almost universal acceptance. Just short of becoming dogma, however, the idea encountered a number of challenges. One centred on the fact that some CTLs turn out not to contain perforin, yet are as lytically active as those brimming over with it^{3,4}. Other investigators found that in many instances degranulation could be blocked in CTLs with little or no effect on target cell lysis, and that lysis could proceed in the absence of Ca^{2+} , which had been demonstrated to be required for perforin polymerization⁵⁻⁷. While proponents of the perforin model struggled to explain away these

apparent inconsistencies, sceptics began to search for alternative lytic mechanisms. But any cytolytic activity seen in CTLs containing even a trace of perforin could never unambiguously be proved not to be due to perforin poking holes in the target cell.

And so it went on. After several years of heated but generally unilluminating debate at meetings and workshops, it became apparent that the only way to resolve definitively the function of perforin in cell-mediated lysis would be to create a perforin-less mouse. Several groups undertook this quest at about the same time, using the technique of targeted gene disruption, but Kägi *et al.* are the first to describe production of such a mouse. Their paper brings perforin's involvement in CTL defences against viral infection to centre stage, and defines some of the questions yet to be answered. The mouse they have generated will allow most if not all of them to be answered.

The first point made strikingly clear by these studies is that perforin is a major factor in the immunopathology generated in mice by the CD8^+ CTL response to lymphocytic choriomeningitis virus (LCMV) injected intracerebrally. Null mutants for perforin fail to overcome such LCMV infections, thereby avoiding the tissue destruction and rapid death resulting from the CTL-led immune attack on cells infected by this relatively benign virus. The existence of LCMV-reactive CD8^+ lymphocytes can in fact be demonstrated in the mice, but they fail either to destroy virally infected cells *in vivo*, or to demonstrate cytotoxicity towards virally infected cells *in vitro* assays. So we can now say unambiguously that perforin plays a major part in the cytotoxic T-cell response to LCMV *in vivo*. In addition to the results with LCMV, Kägi *et al.* show that mice infected with vaccinia virus also fail to develop lytic, virus-specific CTLs.

Successful production of a perforin knockout mouse answers one question that had discouraged some laboratories from even attempting such a project. The only place perforin had been observed outside the immune system is in metrial gland cells of pregnant mice, leading to the suspicion that perforin might be active in pregnancy, and that a perforin knockout mouse might be a developmental lethal. The finding by Kägi *et al.* that null mutant offspring occur in the expected proportion in matings of heterozygous mice proves this fear unfounded. By several criteria, the mice seem to be histologically normal *vis-à-vis* the immune system. The lymphoid tissues are all nor-

mal, as are the CD4/CD8 T-cell ratios.

Intriguingly, the mice generated by Kägi *et al.* are not entirely bereft of cytotoxic function. Although cytotoxicity could not be detected against fibroblast target cells, variable levels of lysis of haematopoietic targets were in fact observed. The source of this cytotoxicity is not yet apparent, but will be of great interest to a number of laboratories. Given that these mice lack perforin completely, it is obvious that punching holes in aberrant cells is only one way for CTLs to kill them. Identifying the nature of this alternative lytic mechanism, and assessing its importance relative to perforin in a wider range of cellular immune reactions, will be greatly facilitated by these mice.

Although the perforin null mutants failed to clear LCMV injected intracerebrally, they were able to clear away allogeneic tumour cells inoculated intraperitoneally. They also appeared to succumb to LCMV inoculated other than intracerebrally. Are these reactions also manifestations of an alternative lytic pathway, or of more generalized cytokine-

induced inflammatory reactions? Further experiments with these mice will doubtless provide the answer. The fact remains that in the case of LCMV intracerebral infection, these other mechanisms, whatever they may be, are insufficient to destroy virally infected cells, and to produce the immunopathology of which this reaction is so classic an example. Clearly, the mice produced by Kägi *et al.* are going to provide us with exciting science for some time to come. □

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1. Kägi, D. *et al.* *Nature* **369**, 31–37 (1994).
2. Henkart, P. A., Millard, P. J., Reynolds, C. W. & Henkart, M. P. *J. exp. Med.* **160**, 75–93 (1984).
3. Berke, G., Rosen, D. & Ronen, D. *Immunology* **78**, 105–112 (1993).
4. Helgason, C. D., Prendergast, J. A., Berke, G. & Bleackley, R. C. *Eur. J. Immun.* **22**, 3187–3190 (1992).
5. Ostergaard, H., Kane, K., Mescher, M. & Clark, W. R. *Nature* **330**, 71–72 (1987).
6. Trenn, G., Takayama, H. & Sitkovsky, M. *Nature* **330**, 72–74 (1987).
7. Lancki, D. *et al.* *J. Immun.* **142**, 416–424 (1989).

VOLCANOES

Rocks of rare refinement

Bruce W. D. Yardley

THE array of natural processes that the geochemist works with includes some that are remarkably effective at taking scarce metals from the most unpromising starting materials — common silicate rocks or melts — and concentrating them up to form ore bodies. Nevertheless, a few of the rarer elements have properties that shadow those of more common elements so closely that nature is unable to separate them: they occur only as minor constituents in minerals dominated by

their more abundant partner.

Rhenium, which is extracted commercially from molybdenum ores, has hitherto been considered a classic example of this type, but on page 51 of this issue Korzhinsky, Tkachenko, Shmulovich, Taran and Steinberg report a remarkable case of element refinement from hot fumaroles near the summit of Kudriav volcano in the Kuril arc, north of Japan. As seen in the photograph, sublimates of pure rhenium sulphide coat the fractured

rock surfaces around the steam vents.

Steam activity around most volcanoes is the result of discharge of ground waters which have circulated to depths of 2 to 4 km, and been heated to temperatures that have probably not exceeded 400 °C. Deeper, hotter rocks are too impermeable for groundwater convection cells to penetrate. But some arc volcanoes emit fumaroles of steam at much higher, magmatic temperatures (1,000 °C or more), and these are probably emissions of primary magmatic steam — steam that has been dissolved in the magma itself, and released as progressive crystallization of anhydrous phases drives the melt composition to water saturation.

Metal-rich sublimates, especially of copper, have been found at high-temperature fumaroles of this type elsewhere in the northwestern Pacific rim and have been identified with the metal-rich fluids that form the primary ore of porphyry copper deposits (R. W. Henley and A. McNabb *Econ. Geol.* **73**, 1–20; 1978), but such an extreme efficiency in metal separation has not been demonstrated previously.

The formation of most hydrothermal ore bodies is the consequence of geological and geochemical processes that cause the solubility of specific metals to change through orders of magnitude over a small physical distance, so that the metals precipitate within a small volume. Changes in temperature, redox conditions or pH are particularly important, and the separation of fluid from magma and the rapid changes in fluid properties near the surface provide a potent natural laboratory, not just for large changes in metal solubility in expanding, cooling steam, but also for selective refinement of specific metals according to the precise sequence of cooling, depressurization, gas loss and chemical interaction with the near-surface environment.

Supercritical fluids, such as carbon dioxide, are being developed for use in industry for their solvent powers (see the News and Views by K. P. Johnston, *Nature* **368**, 187–188; 1994). Natural volcanoes show us not only that steam at magmatic temperatures has the capacity to carry metals in solution, but also that the correct environment of discharge can selectively concentrate specific elements out of dilute solution in this steam. Is this a curious sport of nature, or is there the potential here to develop technologies that are able to concentrate rare metals out of that most mundane of starting materials, the average crustal rock? □

Frank Greenaway

IMAGE
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Left, a rock sample from a fumarole on Kudriav volcano, glistening with silvery flakes of rhenium sulphide (magnified, right).

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No exchange without receipt

Peter Novick and Michelle D. Garrett

SINCE the late 1980s it has been clear that members of the Sec4/Ypt/Rab family of GTPases are key regulators of vesicular traffic. Two groups, Ullrich *et al.*¹ and Soldati *et al.*², have now used *in vitro* Rab-attachment assays to provide direct evidence in support of a long-standing hypothesis³ that guanine nucleotide ex-

change of GDP for GTP (GDP-dissociation inhibitor, GDI, and guanine-nucleotide-exchange factor, GEF) and GTP hydrolysis (GTPase-activating protein, GAP).

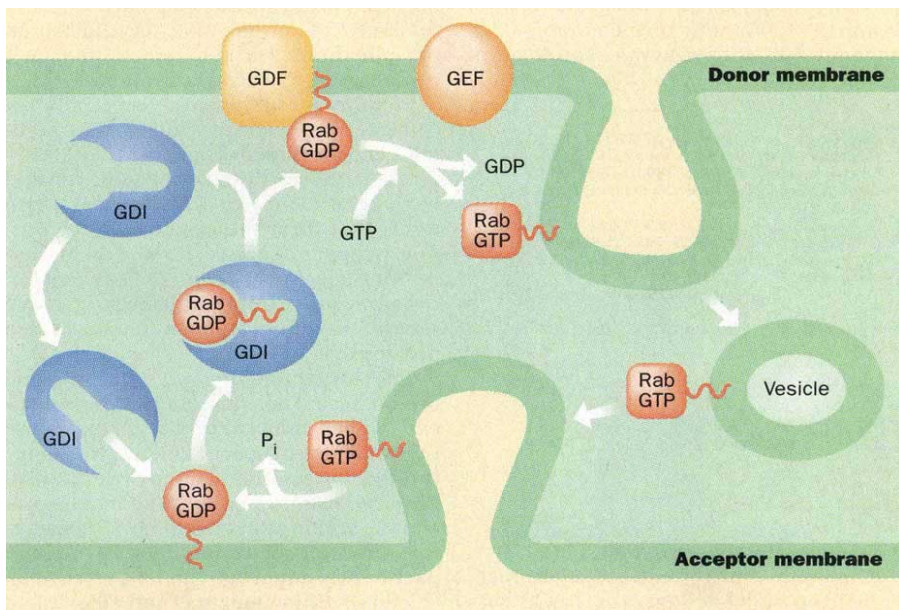
Each Rab protein has a distinct pattern of subcellular localization which may re-

fusion of the vesicle with the acceptor compartment, the Rab protein undergoes GTP hydrolysis and the resulting GDP-bound form is recycled from the target membrane, through the cytosol, back to the donor compartment.

A protein known as rabGDI is thought to have a central role in the recycling pathway. This protein will release Rab proteins from membranes by formation of a soluble heterodimer, but only when the Rabs are in their GDP-bound form⁶. Experiments in yeast have confirmed this role for rabGDI, *in vivo*⁷. The yeast homologue of the gene encoding rabGDI, *GDI1*, was found to be the same as a gene previously defined by a temperature-sensitive secretory mutant, *sec19-1*. When Gdi1p function is lost, the cytosolic pool of Sec4p, a yeast Rab required for post-Golgi export, is depleted. Furthermore, transport is blocked at several stages of vesicular traffic, consistent with the involvement of Gdi1p in recycling of multiple Rabs.

After the Rab protein has been retrieved from the acceptor membrane by rabGDI, the model predicts that the Rab remains soluble until the complex interacts with the appropriate donor membrane, promoting dissociation from rabGDI, membrane attachment and exchange of GDP for GTP. Ullrich *et al.* and Soldati *et al.* address the mechanism of Rab reattachment by establishing *in vitro* membrane-binding assays.

The two groups take similar approaches. A Rab-rabGDI complex is formed from pure components or isolated as a pre-existing heterodimer from cytosol. The complex is then mixed with either isolated organelles or permeabilized cells. The Rab proteins are observed to bind to their appropriate target organelle, while rabGDI dissociates from the Rab protein during the attachment reaction and remains predominantly soluble. As predicted by the model, exchange of GDP for GTP on the Rab protein is coupled to its membrane attachment. But there is a lag of several minutes between Rab attachment and nucleotide exchange, which suggests that the overall reaction requires



A model for Rab cycling. The cycle starts with a Rab protein, in its GDP form, in a complex with its GDP-dissociation inhibitor (GDI) in the cytosol. The Rab-rabGDI complex interacts with a GDI-dissociation factor (GDF), promoting dissociation of the complex and attachment of Rab to the membrane. Membrane-bound Rab interacts with a guanine-nucleotide-exchange factor (GEF), which stimulates release of the bound GDP and binding of GTP. The Rab, now in its GTP form, is incorporated into a carrier vesicle. Following fusion with the acceptor compartment, the Rab hydrolyses its bound GTP and the resulting GDP-bound form is retrieved from the acceptor compartment by rabGDI.

change on Rab proteins is coupled to their attachment to distinct membrane compartments. One paper was published in March¹; the other appears on page 76 of this issue².

Vesicular transport is used by eukaryotic cells to transport material from one organelle to another. A subset of the components of the donor organelle is packaged into small carrier vesicles which, on fusing with the target organelle, complete the selective transfer of cargo. Elaborate cellular machinery is required to control the processes of vesicle budding, target organelle recognition and vesicle fusion. One class of proteins involved is the Ras-related Rab GTPases. Like all members of the Ras superfamily, these proteins can stably exist in either GDP- or GTP-bound conformations because they have very low intrinsic rates of GTP hydrolysis and GDP dissociation. The switch in conformation is regulated

by factors which control the rates of exchange of GDP for GTP (GDP-dissociation inhibitor, GDI, and guanine-nucleotide-exchange factor, GEF) and GTP hydrolysis (GTPase-activating protein, GAP). Each Rab protein has a distinct pattern of subcellular localization which may reflect its function at a particular stage of membrane traffic. Ullrich *et al.* and Soldati *et al.* looked at Rab5 and Rab9 respectively. Rab5 associates with the plasma membrane and early endosomes, and regulates endocytic transport from the cell surface⁴; Rab9 regulates transport from late endosomes to the Golgi apparatus and is found on both compartments⁵. Membrane association is dependent upon covalent addition of the 20-carbon lipid geranylgeranyl to one or two carboxy-terminal cysteine residues. However, prenylated Rab proteins are also found in the cytosol.

The current view is that Rab proteins undergo a cycle of localization coupled to the cycle of nucleotide exchange and hydrolysis (see figure). By this model, Rabs bind to the donor compartment in a reaction that is coupled to the exchange of GDP for GTP. The GTP-bound form is incorporated into vesicles. Following

1. Ullrich, O., Horiuchi, H., Bucci, C. & Zerial, M. *Nature* **368**, 157–160 (1994).
2. Soldati, T., Shapiro, A. D., Svejstrup, A. B. D. & Pfeffer, S. R. *Nature* **369**, 76–78 (1994).
3. Bourne, H. *Cell* **53**, 669–671 (1988).
4. Bucci, C. *et al.* *Cell* **70**, 715–728 (1992).
5. Lombardi, D. *et al.* *EMBO J.* **12**, 677–682 (1993).
6. Araki, S., Kikuchi, A., Hata, Y., Isomura, M. & Takai, Y. *J. Biol. Chem.* **265**, 13007–13015 (1990).
7. Garrett, M. D., Zahner, J. E., Cheney, C. M. & Novick, P. J. *EMBO J.* **13**, 1718–1728 (1994).
8. Chavrier, P. *et al.* *Nature* **353**, 769–772 (1991).
9. Brennwald, P. & Novick, P. *Nature* **362**, 560–563 (1993).
10. Moya, M., Roberts, D. & Novick, P. *Nature* **361**, 460–463 (1993).
11. Burton, J., Roberts, D., Montaldi, M., Novick, P. & De Camilli, P. *Nature* **361**, 464–467 (1993).
12. Burnstein, E. S. & Macara, I. G. *Proc. natn. Acad. Sci. U.S.A.* **89**, 1154–1158 (1992).
13. Boguski, M. S. & McCormick, F. *Nature* **366**, 643–654 (1993).

at least two steps. The first involves recognition by a component, termed GDI-dissociation factor (GDF), which causes dissociation of the Rab-rabGDI complex and thus promotes binding of the Rab protein to the membrane. The second is interaction of the membrane-bound Rab with a guanine-nucleotide-exchange protein that catalyses release of GDP and binding of GTP. The GDF and GEF activities could be mediated by one protein, by two distinct proteins, or by different subunits of a protein complex.

Which activity confers the specificity of attachment? An obvious candidate is the GDF. This would suggest that there may be a different GDF for each Rab, and that different GDFs are associated with different compartments. One might also predict that the GDFs interact with the hypervariable domain at the carboxy termini of the Rab proteins, as this region is a targeting sequence^{8,9}.

Testing these predictions will require purification of the proteins that regulate attachment and exchange and cloning of the genes. Candidate Rab exchange proteins have been identified through genetic screens in yeast^{10,11} and biochemical analysis of brain extracts¹², and have been shown to stimulate the dissociation of GDP from Rab proteins in solution. Perhaps these proteins, or their relatives, are required for the exchange reaction that occurs on the membrane surface. It will be interesting to see whether the Rab exchange reactions are subject to the elaborate regulation that is typical of other members of the Ras superfamily¹³. □

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DEVELOPMENTAL BIOLOGY

Catch of the decade

Jean-Paul Concordet and Philip Ingham

How good is the zebrafish, *Danio rerio*, as a model organism for analysing vertebrate development? Proponents of the fish have made much of its advantages — its small size, rapid life cycle, the optical clarity and beauty of its embryos; moreover, it offers the option of inducing haploid development¹. Sceptics, by contrast, have questioned the legitimacy of using a lowly teleost as a model for man, at the same time highlighting its technical drawbacks. In particular, they have pointed to the difficulties inherent in proceeding from mutant phenotype to molecular characterization of a gene, a problem obviated in mice by reverse genetics methods.

The growing realization that all animals are constructed along remarkably similar lines² has done much to undermine the first objection, but doubts have persisted over the effectiveness of mutant screening as a means of identifying new genes of developmental importance. A trio of papers in *Current Biology*³, *Genetics*⁴ and last week's *Science*⁵ should now convince even the most sceptical that the zebrafish is here to stay in developmental biology. The first two describe the establishment of conditions for the efficient induction and recovery of embryonic lethal mutations; the third reports the use of anonymous molecular markers, generated by the polymerase chain reaction (PCR), to localize mutations and construct a genetic linkage map that will

facilitate the molecular cloning of genes identified in mutant screens.

The key to success in the genetic analysis of the embryonic development of any organism is to perform saturation screening^{6,7}, and the key to successful saturation screening is efficiency. What



Model potential — the zebrafish, *Danio rerio*.

are required are efficient mutagenesis procedures that maximize the number of lethal hits per genome, and efficient screening procedures that minimize the time and effort involved in identifying these hits. Just as Christiane Nüsslein-Volhard, with Eric Wieschaus, revolutionized the genetic analysis of *Drosophila* development by devising ways of screening large numbers of mutagenized flies, she³ and her former colleague, Wolfgang Driever⁴, have now streamlined the generation, maintenance and screening of

large numbers of mutagenized fish.

Ironically, the production of haploid embryos, originally advocated by George Streisinger as the best way of screening for recessive mutations, has been adjudged too unreliable and labour intensive — haploids have to be generated by *in vitro* activation of eggs obtained from individually squeezed female fish, and a significant proportion of them develop abnormally, hindering identification of mutant phenotypes. Instead, the two groups^{3,4} have chosen to let the fish do the work, generating diploid homozygous mutant embryos by natural spawning. This means growing the F₂ generation of fish to adulthood and screening their progeny for lethal phenotypes; because the zebrafish, unlike *Drosophila*, still has no good visible markers that allow mutagenized chromosomes to be tracked, several single-pair matings must be established within individual families of fish to ensure homozygosity of the newly induced mutations. So screening 1,000 genomes will typically entail growing some 30,000 fish and setting up 10,000 pair matings. Hence the need for a smooth-running aquarium facility.

When this number of fish is involved, it is obviously essential that the induced mutation rate be as high as possible — and, equally important, that induced mutations should be efficiently transmitted through the germ line. To satisfy these criteria, the Nüsslein-Volhard and Driever laboratories have undertaken an extensive investigation of the parameters underlying mutagenesis of the male germ line by two widely used alkylating agents, ethyl nitrosourea (ENU) and ethyl methyl sulphonate (EMS). Although both mutagens are highly efficient at inducing mutations in the DNA of spermatozoa and mature sperm, EMS is considerably less effective in mutating spermatogonial DNA. This is probably because these cells can repair the lesions introduced by EMS but not those caused by ENU.

This is important because the lesions induced in post-mitotic sperm cells are present in only one strand of the DNA, and hence give rise to individuals that are mosaic for the mutated gene; because the contribution of mutant-carrying cells to the germ line of such mosaic individuals is usually low, few, if any, of these mutations are recoverable in the next generation. By contrast, mutations induced in the DNA of spermatogonia are fixed following cell division, and give rise to sperm that produce non-mosaic animals which transmit the induced mutation in a Mendelian fashion. Accordingly, ENU has been adopted as the mutagen of choice by both groups, and conditions yielding a single-

Max Gibbs/Oxford Scientific Films

locus hit frequency of $0.2-2 \times 10^{-3}$ (similar to that found in mice) have been established.

The efficiency of mutagenesis was assessed by two criteria. First, new mutations at previously known loci affecting pigmentation were scored. Second, after inbreeding, new recessive lethal mutations were identified by simple visual inspection of embryos during early development; screening the equivalent of 240 F_2 lines yielded a total of 296 ENU-induced mutations, equivalent to a rate of 1.2 lethal hits per genome. Taken together with the single-locus hit rate estimated from the new pigmentation mutant alleles, these results yield a crude estimate of 600–1,500 as the number of genes that will mutate to give a visible phenotype. The two laboratories have therefore set out to screen 3,000 to 4,000 F_2 families each, and the hundreds of mutants found should reveal about 90 per cent of all embryonic lethal genes. Given the logistics now in operation, the screen should be completed within only two years.

The catch of mutants will be good, but what will it hold? Nüsslein-Volhard's group reports a phenotypic classification of the mutants isolated in a pilot screen.

Most (70 per cent) of them exhibit rather nondescript defects, such as swollen heart cavities or other general abnormalities. The remaining 30 per cent are more promising: some show defects in specific structures that arise early in development, such as the notochord, suggesting that the genes responsible may be involved in early patterning events, whereas others affect the later differentiation of structures such as eyes, ears, jaws or heart. Even in the absence of any molecular characterization of the mutated genes, such embryonic lethal mutants can serve as powerful tools in the analysis of early development. The ability to generate genetic mosaics by cell transplantation between wild-type and mutant embryos has provided insights into the cellular basis of processes such as cell migration and inductive interactions, as exemplified by studies of the *spadetail* and *cyclops* mutants^{8,9}. Ultimately, however, the goal must be to understand these processes at the molecular level, and for this purpose the cloning of the mutated genes will be essential.

A step in that direction has now been taken by John Postlethwait and co-workers at the University of Oregon⁵. Following strategies developed in plant genome analysis, several hundred decamers were screened for their ability to

PCR amplify sequences specific to either of two zebrafish strains, AB and Darjeeling. Postlethwait's group thereby identified 402 randomly amplified polymorphic DNA (RAPD) sequences and examined their segregation in 94 haploid offspring of an AB/Darjeeling female. These data were used to construct the first linkage map of the zebrafish genome. The ability to produce haploid offspring proved crucial in this exercise because it circumvents the need for inbreeding in inferring genotype from phenotype. The molecular basis for RAPD polymorphisms is uncertain, and so difficulties in reproducing adequate conditions for PCR using decamers may impede the use of the map as it stands. But cloning RAPD sequences should allow the design of longer primers with higher specificity to convert them into sequence-tagged amplified regions.

Using this same strategy, the Oregon group have mapped a recessive pigmentation mutation, *brass*, again exploiting the ability to genotype haploid embryos directly. They analysed the segregation of RAPD sequences between phenotypically mutant and wild-type haploid offspring of a heterozygous *brass*/Darjeeling female, and identified several markers that are closely linked to the mutation. Such mapping can be done quite quickly, and Postlethwait and colleagues estimate that, using 1,000 primer sets, any mutation can potentially be mapped to within 0.5 centimorgan of a molecular marker (which, according to their estimates, means to within 300 kilobases). Such closely linked markers can then be used as starting points for cloning the mutant gene by chromosomal walking. A physical map of the zebrafish genome similar to that developed for humans would greatly increase the speed of this step.

Given the success of the systematic genetics approach in *Drosophila*, expectations of the zebrafish screens are high. Many unknowns remain, but the development of large-scale screens and of the genetic linkage map surely mark the dawning of zebrafish genetics. □

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In the wake of Columbus

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Mary Evans/Explorer

If only Christopher Columbus had trailed a thermometer behind the *Santa María* instead of a tender, he could have extended oceanographic temperature records by several centuries. The lack of decent time series is one of the great problems for oceanographic science; today's detailed, worldwide monitoring is a fairly recent development. But Columbus's voyage of discovery in 1492 was at least of indirect benefit to oceanographic science — celebratory voyages along the route taken by the *Pinta*, *Niña* and *Santa María* (shown above) make it one of the most thoroughly sampled ocean transects. On page 48 of this issue, Gregorio Parrilla and co-workers add the results of a 1992 Atlantic crossing, made as part of the 500th anniversary celebrations, to those of two oceanographic expeditions in 1957 and 1981. In combination, the three records show a persistent warming between depths of 800 and 2,500 m, together with intriguing hints of large-scale spatial variation. Could this be a signal of climate change? Alas, things are unlikely to be quite that simple, because global warming would be expected to manifest itself in the surface waters rather than at depth.

Lindsay Matthews

1. Streisinger, G. C., Walker, C., Dower, N., Knauber, D. & Singer, F. *Nature* **291**, 293–296 (1981).
2. Slack, J. M. W., Holland, P. W. H. & Graham, C. F. *Nature* **361**, 490–492 (1993).
3. Mullins, M. C., Hammerschmidt, M., Häffter, P. & Nüsslein-Volhard, C. *Curr. Biol.* **4**, 189–202 (1994).
4. Solnica-Krezel, L., Schier, A. F. & Driever, W. *Genetics* **136**, 1401–1420 (1994).
5. Postlethwait, J. H. *et al.* *Science* **264**, 699–703 (1994).
6. Nüsslein-Volhard, C. & Wieschaus, E. *Nature* **287**, 795–801 (1980).
7. Mayer, U., Torres Ruis, R. A., Berleth, T., Misera, S. & Jürgens, G. *Nature* **353**, 402–407 (1991).
8. Ho, R. & Kane, D. *Nature* **348**, 728–730 (1990).
9. Hatta, K., Kimmel, C., Ho, R. & Walker, C. *Nature* **350**, 339–341 (1991).

Pressure-cooking Triton

Jonathan I. Lunine

THE quest to understand the origin of our planetary system takes one from meteorite samples in laboratories to the outermost reaches of the Solar System. There, relatively primitive bodies exist whose composition, in the optimist's view, can be compared with the original gas and dust from which the planets sprang. But the histories of such bodies may, it seems, be far from simple. Studies of Neptune's satellite Triton, from the ground and through the extraordinary 1989 flyby of Voyager 2, indicate a composition distinct from that of interstellar clouds and comets. Theoretical work points to an early history distinguished by strong tidal heating and melting of its interior.

Now Shock and McKinnon¹ have drawn these two facets together, in a model that ties the modification of Triton's composition directly to its early period of heating. Moreover, they predict the synthesis within Triton of as yet unobserved organic molecules which on Earth may have played key roles in the chemistry that led to life's origin.

Cold start

In the outermost Solar System — roughly speaking, the region beyond the orbit of Uranus — conditions were probably cold enough during planetary formation to condense and partially preserve the ices of water and more volatile molecular species produced in the molecular cloud from which the Solar System formed. This record, then, is complementary to that of the meteorites, whose parent objects formed closer to the Sun, preserving the rock and involatile carbon record but not the ices. Of particular interest are the carbon- and nitrogen-bearing molecular species, because these elements are common in the Galaxy and are observed in molecules in star-forming clouds. The differences in molecular abundances between such clouds and objects in the outer Solar System provide a measure of the chemical and physical processes that have taken place during and after the system's formation.

Within molecular clouds, carbon monoxide is the main carbon-bearing molecule, followed by methane, carbon dioxide and low-carbon-number organics². Much of the interstellar carbon is locked in the solid phase in diverse species ranging from graphite to these more volatile organics. The inventory of nitrogen-bearing species is less clearly understood, but molecular nitrogen certainly dominates in the gas and probably in solid phases as well. Ammonia may constitute a few per cent of the total nitrogen in the solid phase. The ratio (CO/N₂) was almost

certainly unity or higher in the molecular cloud that collapsed 4.5 billion years ago to form the Solar System.

The inventory of the outer Solar System is equally diverse. The atmospheres of the giant planets, analysed from ground-based and space-borne spectroscopy, show primarily methane and ammonia in the carbon- and nitrogen-bearing phases, indicating nearly complete re-equilibration under the influence of the high temperatures and pressures of their immense interiors, and the predominance of molecular hydrogen which drives the chemistry toward the reduced state.

Comets are another matter. They are likely to have undergone little processing since formation. Indeed, they seem to have abundances of carbon- and nitrogen-bearing species similar to those in molecular clouds, in particular showing a dominance of CO over other carbon-bearing molecular species, as well as over N₂. So might they be an accessible sample of partially preserved primordial grains³? The difficulty is that it is unclear where the comets formed in relation to the rest of the Solar System — proposals range from the Uranus-Neptune region all the way to interstellar space. It is therefore difficult to use comets by themselves as a chemical touchstone for the origin of planets. But their chemical record can usefully be compared with other objects in the outer Solar System whose birthplace is better known.

Enter Triton and Pluto. Both probably formed in solar orbit close to where they are today, and almost certainly within a zone known as the Kuiper belt, from 30 to 50 astronomical units from the Sun (1 AU is the distance from Earth to Sun), which may contain many such objects⁴. Triton's currently retrograde orbit (backwards compared with most planets and moons) around Neptune is best explained as the result of capture from a solar orbit similar to Pluto's into a loosely bound orbit around Neptune, either by gas drag (W. B. McKinnon and A. C. Leith, manuscript in preparation) or collision with an existing satellite⁵. The subsequent evolution of the orbit towards the more tightly bound, circular path observed today was accompanied by dissipation of the orbital energy within Triton ('tidal heating'), enough to melt the water ice in the satellite.

Pluto, although modestly heated during formation of its moon Charon, probably did not suffer as much heating as Triton. Thus the molecular compositions of two objects, similar in size, bulk density and region of formation, present a special opportunity to understand the evolution

of volatiles during the early history of the outer Solar System.

Ground-based spectroscopy of Triton and Pluto^{6,7}, and data returned during the Voyager 2 flyby, show that the surface compositions differ considerably from those of comets and molecular clouds. On both Triton and Pluto, CO is much less abundant than N₂. On Triton, CO is also less abundant than CO₂, which is not detected on Pluto. The dominant species lying on the surface is N₂.

From the bulk densities of Triton and Pluto, and the cosmic abundances of elements, it seems likely that their interiors are largely silicates and water ice. The molecular ices exposed to spectroscopists are a veneer which is most plausibly derived from outgassing of the interiors. Arguably the abundances seen on the surface are a fair reflection of the original relative abundances of these ices. This is particularly so for CO and N₂, whose volatility and molecular properties are so similar that any outgassing could fractionate them only slightly. The case is different for CO₂, which is harder to outgas because it is less volatile, so the surface predominance of CO₂ over CO on Triton should reflect an even greater predominance in the interior and hence an even greater difference from comets and molecular clouds.

The 'missing CO' problem is a striking one, and suggests a good deal of chemical evolution in the early history of Triton and Pluto. Simple explanations have proved elusive. Arguments for extensive chemistry in the gas that formed the solar nebula, distinct from that in the original molecular cloud, still come up with CO as the predominant carbon molecule⁸. Loss of carbon monoxide from Triton and Pluto early in their history is plausible, given their low gravities and (especially for the former) their warmth. But it fails to differentiate between CO and N₂, and any such model must postulate an additional source of N₂ to make up for early losses. Production of N₂ from NH₃, which is a much less volatile yet reasonably abundant nitrogen-bearing molecule, is plausible, but an early Triton or Pluto environment which could support such a chemistry has yet to be well established.

Deep heat

Shock and McKinnon¹ have developed an alternative and elegant explanation for Triton, which draws on an initial proposal by Stevenson and Gandhi⁹. The tidal heating suffered early on was probably sufficient to melt Triton's water ice, resulting in an interior composed of a vast 'mantle' of liquid water overlying a 'core' of silicates and some iron. At the boundary between the mantle and core, liquid water would have circulated through the rock, in analogy to the hydrothermal circulation seen at the mid-ocean ridges of

the Earth's oceans. The combination of tidal heating and that due to the decay of radioactive elements expected in the rock would have provided temperatures of at least 200 °C; the pressure at the interface between core and mantle can be estimated at a few thousand bars.

This pressure-cooker would have been in operation for a hundred million years or so, during which time the carbon- and nitrogen-bearing molecules dissolved in the water could have undergone very extensive chemical reworking. Stevenson and Gandhi⁹ proposed that CO might be chemically destroyed in such a system. Shock and McKinnon have undertaken quantitative examination of the products of this chemistry.

Their model considers 18 compounds of the elements C, N, O and H, and calculates their equilibrium abundances under the assumption that Triton's hydrothermal system is buffered by particular assemblages of rocks, which determine the redox state of the chemical system. The most striking of their results is that, for a very wide range of redox states of the liquid water (corresponding to a range of possible mineral assemblages), CO is a minor species relative to CO₂. That is, cooking a mix of carbon-bearing species in aqueous solution at high temperature and pressure will substantially decrease the carbon monoxide abundance.

The model further predicts that N₂ is more abundant than CO over a wide range of plausible redox states. For progressively more reducing mineral assemblages, N₂ does give way to NH₃ as the predominant nitrogen-bearing species, and eventually no longer dominates over CO. However, the range over which the N₂ dominates is large, so chemical alteration in a hydrothermal system appears capable of producing the carbon and nitrogen inventories seen on Triton's surface.

A number of questions are raised by the work. The abundance of CH₄ is a particularly problematic one, as that molecule is seen on the surfaces of both Triton and Pluto. Shock and McKinnon exclude methane from the chemical system, arguing that in terrestrial hydrothermal systems it does not readily equilibrate with the other compounds except at rather high temperatures. In effect, CH₄ and other light hydrocarbons are kinetically inhibited from participating in the chemistry. This may well be the case in terrestrial hydrothermal systems, but the relevant dynamical time constants in Triton's system could be different.

A second issue is how these materials find their way to the surface, and the timing of surface extrusion relative to hydrothermal cycling. The carbon- and nitrogen-bearing ices must be extruded after most of the mantle material has been hydrothermally altered, which sets constraints on the timing of outgassing,

volcanic extrusion, and mantle cooling and refreezing. These are not addressed in detail in the paper, but will be grist to the mill of planetologists who model such processes.

Pluto's composition represents a third question raised by this model. The inventory of CH₄ and CO on Pluto is modestly larger than on Triton, and from the lack of detection of CO₂ one can estimate an upper limit which is roughly a third of that on Triton. Thus one might argue, and the authors do, that Pluto has been subjected to less hydrothermal processing than has Triton. Not much less, though, because CO remains a very minor component relative to N₂. So if hydrothermal processing has determined the surface inventory on both bodies, Pluto too must once have had a liquid water mantle. This sets constraints on Pluto's thermal history, arguing that in spite of the absence of tidal dissipation Pluto must have undergone substantial heating. Perhaps we must face the fact that neither object represents an unaltered sample of the primitive material of the outer Solar System.

As do all good models, Shock and McKinnon's story leaves us with an intriguing punchline. Hydrothermal alteration acts not just to destroy CO but also to produce a suite of organic compounds of biological interest, including methanol, formic acid, urea and glycine, with abundances dependent on the buffering mineral assemblages.

The lack of spectroscopic detection of such species on Triton and Pluto may reflect the redox state of the internal chemistry, their failure to be erupted on the surface, burial by more volatile ices, or just the difficulties associated with spectroscopic examination of distant, dim bodies. Nonetheless, their presence in the theoretical soup invoked for Triton's interior reinforces the fact that many of the raw materials for life can be made in many places in the cosmos. Hydrothermal alteration itself probably occurred in the parent bodies of asteroids and hence meteorites, producing organic compounds which found their way to the Earth in the past, and continue to do so. □

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1. Shock, E. L. & McKinnon, W. B. *Icarus* **106**, 464–477 (1993).
2. Van Dishoeck, E. F. et al. in *Protostars and Planets III* (eds Levy, E. H. & Lunine, J. I.) 163–241 (University of Arizona Press, Tucson, 1993).
3. Mumma, M. J. et al. in *Protostars and Planets III* (eds Levy, E. H. & Lunine, J. I.) 1177–1252 (University of Arizona Press, Tucson, 1993).
4. Stern, S. A. *Icarus* **90**, 271–281 (1991).
5. Goldreich, P. et al. *Science* **245**, 500–504 (1989).
6. Cruikshank, D. P. et al. *Science* **261**, 742–745 (1993).
7. Owen, T. C. et al. *Science* **261**, 745–748 (1993).
8. Lewis, J. S. & Prinn, R. G. *Astrophys. J.* **238**, 357–364 (1980).
9. Stevenson, D. J. & Gandhi, A. S. *Lunar planet. Sci.* **XXI**, 1202–1203 (1990).

Groaning boards

THE traditional coal mine used wooden roof supports. If a roof fall threatened, they began to creak: a useful early warning. Wood creaks under load because individual fibres start to slip jerkily past one another. Similarly, metallic tin 'cries' when bent. One small volume of the lattice dislocates under stress; this increases the load on its neighbours and they yield in turn. So dislocations are triggered in sudden cascades. Daedalus reckons that every material must creak out its own overload warning in this way. Sadly, such warnings are usually too high to hear.

Daedalus is now listening to them. He is sticking piezoelectric contact microphones onto bars of test material, and then bending or stretching them in various ways. The microphone output goes to a frequency shifter, of the type used by biologists to listen to the ultrasonic cries of bats. Once converted to audio, the creaks and groans of each specimen should reveal its state of stress, and how close it is to failing.

With luck, a specimen will emit a characteristic cry for each type of applied stress. When that stress is relaxed, internal hysteresis will return the lattice to its original shape by a slightly different route, and it will emit a different return cry. Once Daedalus has cracked the code, any engineering structure could be fitted with microphones. It would thereafter provide a running commentary on its changing conditions of stress.

Aerospace engineers will be the first beneficiaries. A few microphones, each picking up the cries of a wide volume of structure, should give total surveillance of even a big aircraft. The system will be calibrated by known loads; thereafter computerized signal analysis will easily locate and disentangle the simultaneous chatter of many different spars and panels. Usually the groans of relaxation will alternate reassuringly with the creaks of stress. The shriek of brief overload, the crackle of plastic flow, the insidious muttering of advancing creep and fatigue, will echo in from the most distant and inaccessible points. A microphone on a turbine shaft might even detect creep murmurings transmitted from the blades themselves.

Engineers will rush to fit Daedalus's system to structures old and new. A few microphones will monitor them more comprehensively than the densest web of strain gauges. In countries such as Britain, it will allow elderly cranes, Victorian bridges and crumbling infrastructure generally to be patched up and kept going exactly to the point of collapse.

David Jones

Ozone profiling using kites

SIR — An innovative technique for profiling ozone concentration in the lower atmosphere was begun last August as part of the North Atlantic Regional Experiment (NARE) campaign in Nova Scotia. The technique involved repeatedly transporting a conventional ozonesonde package up and down the tether of a high-tech kite 'platform' stationed at altitudes up to a few kilometres. This technique enabled us to obtain a rapid series of vertical profiles of temperature, pressure, humidity and ozone concentration using the same sonde package.

Although kites have been used for atmospheric research since the mid-1700s, their potential as research tools has been improved substantially by the advent of lightweight, high-strength materials such as Kevlar (for tethers) and Mylar (for kites) (B. B. Balsley *et al.* *Bull. Am. Met. Soc.* **73**, 17–29; 1992). The NARE kite system consisted of a 15 m² cross-section Mylar kite, a 7,500-m reel of 280 kg test Kevlar, a gasoline-powered hydraulic winch, a payload consisting of a conventional Vaisala-sonde (temperature, pressure and humidity) coupled to a Science Pump Corporation ozonesonde, and our WindTRAM (see below). The total system weight was approximately 32 kg.

A specially designed WindTRAM (Tethered Rover for Atmospheric Measurements) transported the ozonesonde payload up and down the tether. The TRAM (photograph) comprised a 1-m² wind-driven device attached to the tether via a long cylindrical tube, and provided sufficient lift to loft approximately 5-kg payloads rapidly from the ground to the kite. The TRAM was brought down either by radio command from the ground station, or by a mechanical release that operated if the TRAM approached too close to the kite itself.

Two sets of kite-borne profiles are presented in the figure. Examination of this figure shows the excellent height resolution available from the kite-borne technique. The narrow (20 m) enhanced layer at about 850 m in the first profile exemplifies this capability. Note also the associated 'kink' in the potential temperature profile and the humidity profile at the same height.

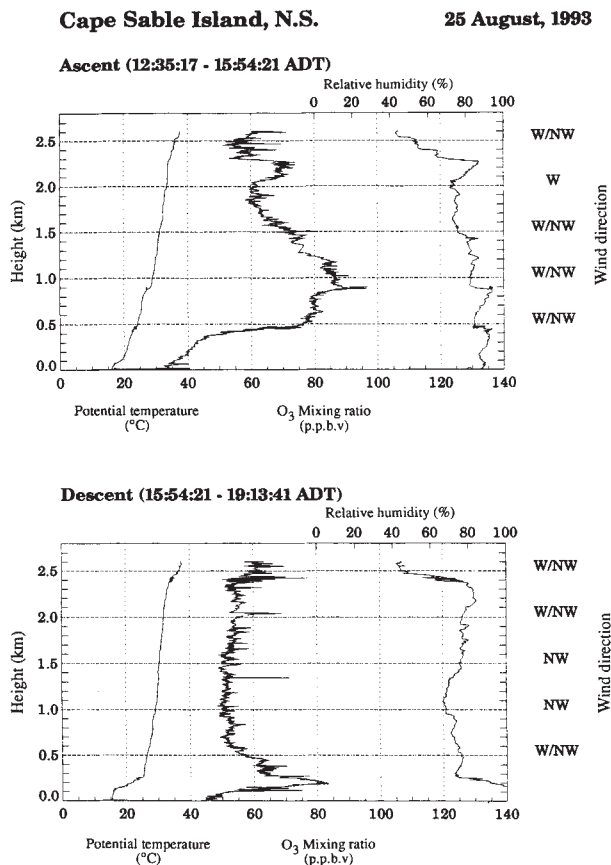
A strong temporal variability of the ozone profile can be inferred by intercomparing these two profiles, which were separated by about 3 h. The enhanced ozone concentration lying between roughly 500 and 1,500 m has all but disappeared by the time the sonde has retraced through the region about 3 h later. Since the time to make each profile is comparable to the time separation between profiles, it is apparent that neither of these profiles represents a true instantaneous profile. Much higher time resolution is needed to determine the time evolution of the profiles. This require-

ment is well within the capability of the WindTRAM, which has a measured rise rate at least an order of magnitude larger than that used in the current examples.

We obtained about 40 profiles of temperature, pressure, humidity and



Photograph of the WindTRAM connected to the kite tether. The WindTRAM is designed to carry the sonde payloads up and down the tether while the kite remains aloft.



Profiles of temperature, humidity and ozone mixing ratio obtained by connecting the ozonesonde directly to the tether and reeling the kite out (a, ascent profiles) and back in (b, descent profile). As indicated, these two profiles took roughly 3 h each to measure, and are separated in time by about the same amount. Approximate wind directions appear to the right.

ozone concentration between 8 and 29 August 1993. During this period, the measured ozone concentration aloft depended strongly on the direction of the prevailing wind. The largest values were obtained when the wind was coming from the west, from the northeastern United States. Note that values aloft were not well correlated with concurrent ground-based values; it was not uncommon for the ground-level mixing ratio of ozone to be 20–40 p.p.b.v., while mixing ratios above about 300 m were in the 90–130 p.p.b.v. range.

These results underscore the practicality of the reuse of conventional ozonesondes using a kite-borne technology. Perhaps of greater importance is the demonstrated feasibility of reasonably continuous profiling of any number of atmospheric constituents using reusable sondes carried aloft by this technology. Primary requirements are that the instrumentation be lightweight (a few kg) and consume minimal power (a few watts). Finally, our theoretical studies suggest the possibility of kite profiling into the lower stratosphere.

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Support for dental HIV transmission

SIR — On the basis of a phylogenetic analysis of HIV sequences, *Ou et al.*¹ concluded that a Florida dentist infected five of his eight known HIV-1 seropositive patients. These authors used bootstrap resampling² to test the reliability of their finding, and found that the HIV sequences from the dentist and infected patients formed a monophyletic group in 79% of the replicates in parsimony analysis. *DeBry et al.*³ in *Scientific Correspondence* questioned the conclusion of dental transmission, however, because a bootstrap analysis (based on threshold parsimony) of independently sequenced HIV variants clustered only one of the patient sequences with a dental sequence in the majority-rule consensus tree. *DeBry et al.* concluded that their analyses "...show that the available data are consistent with both the dental transmission hypothesis and the null hypothesis (the patients were independently infected from the local community) and do not distinguish between the two." But both studies^{1,3} used an analysis of the bootstrap results that may not be the most appropriate method for this case. We have re-analysed the two datasets, as well as sequences from new patients and new local controls, and find strong support for trees consistent with HIV transmission between the dentist and six of ten of his seropositive patients.

Both *Ou et al.* and *DeBry et al.* used the standard systematic practice of summarizing the bootstrap results by showing the percentage of time particular clades were found in a majority-rule consensus tree of the replicates². Both studies considered patient HIV sequences that clustered more closely with the dental sequences than with any local controls to be consistent with the dental transmission hypothesis. However, given that the question of dental transmission is asked separately for each patient, the majority-rule consensus tree does not summarize the appropriate information. Individual patient sequences

may be consistent with the dental transmission hypothesis in every bootstrap replicate, and yet not be present in the majority-rule consensus tree. To demonstrate this fact, consider an hypothetical example (shown in the figure) in which the majority-rule consensus tree provides no support for any of the patient sequences to group with the dental sequence. However, all of the trees are consistent with the dental transmission hypothesis for the sequence from patient A (because the sequences from patient A are always more closely related to those from the dentist than to any of the local controls). Clearly, then, the majority-rule consensus tree is not adequate for identifying the patients who may have been infected by the dentist. Whether or not a tree is consistent with the dental hypothesis for any given patient should be dependent only on the placement of the patient sequence with respect to the dental sequences and local controls, not other patients.

An alternative method of summarizing the bootstrap results poses the question of the dental transmission hypothesis separately for each patient. Thus, for the example above, the support is 100% for patient A, 67% for patients B and C, and 0% for patient D. This is a more appropriate way of summarizing the results, because there is no *a priori* way to hypothesize the extent of the 'dental clade' and the dental transmission hypothesis should be tested for each patient separately. The table shows the results of such an analysis, based on reanalyses of refs 1 and 3, and a new dataset collected by the Centers for Disease Control and Prevention (CDC; Marcia Kalish, personal communication). The

latter dataset is the same as that analysed by *Ou et al.*, but includes sequences from three new patients as well as five local controls from the same county as the dental practice. The results in the table show that all the datasets support the conclusions of *Ou et al.* even more strongly than was originally suggested, with the exception of patient G in the *DeBry et al.* dataset (which shows lower support than the 79% bootstrap propor-

INDIVIDUAL BOOTSTRAP SUPPORT (BASED ON 1,000 REPLICATES) FOR THE DENTAL TRANSMISSION HYPOTHESIS FOR SEQUENCES FROM 10 PATIENTS (A–J).

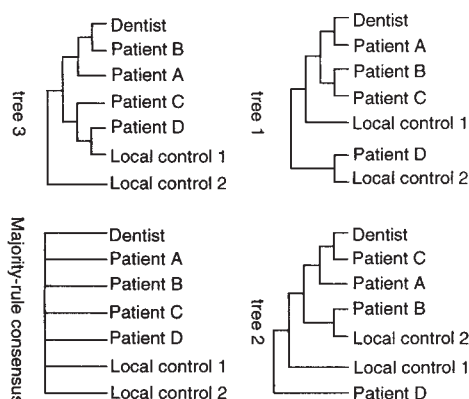
Patient	Other HIV risk factors*	Data set		
		Ou <i>et al.</i> BP (n)	DeBry <i>et al.</i> BP (n)	New CDC data BP (n)
A	No	83–99 (2)	89–98 (4)	91–100 (2)
B	No	78–79 (2)	89–90 (3)	84–86 (2)
C	Unconfirmed	98 (2)	88 (4)	99 (2)
D	Yes	1 (2)	Not examined	1 (2)
E	No	79 (2)	94–95 (4)	86 (2)
F	Yes	0 (2)	0 (2)	0 (2)
G	No	94–96 (2)	63–65 (2)	96–98 (2)
H	Yes	Not examined	Not examined	8 (1)
I	No	Not examined	Not examined	89 (1)
J	Yes	Not examined	Not examined	0–13 (2)

* Sources: refs 4, 10; Carol Ciesielski, Centers for Disease Control and Prevention, personal communication.

In each study, multiple sequences were analysed from most individuals; the range of bootstrap proportions (BP, given as a percentage) among sequences is shown for each individual, with the number of sequences examined shown in parentheses. The *Ou et al.* dataset¹ includes six local control sequences (the local control consensus sequence was not used here because it does not represent an individual viral sequence), two dental sequences and an outgroup. The *DeBry et al.* dataset³ includes 13 local control sequences (from 10 individuals) and six dental sequences. The new CDC dataset includes all the earlier data from ref. 1 plus five new local control sequences (see text). Parsimony analyses were based on unordered characters with all state changes weighted equally⁹ (information on analysis based on alternative weighting and ordering assumptions, as well as details of the analyses and searching methods, is available on request from D. M. H.).

tion suggested by *Ou et al.*). The lower consensus bootstrap values reported by *DeBry et al.* appear to be largely the result of the more ambiguous placement of patient G among their trees; three of the purportedly infected patients (A, B, E) actually cluster more strongly with the dentist in the *DeBry et al.* dataset than in the original study. *Ou et al.* concluded that their tree was consistent with the dental transmission hypothesis for patients A, B, C, E, G; at best, the *DeBry et al.* data cast doubt only on patient G. Our analyses of the new CDC data support the original conclusion, and also include patient I in the dental clade (in agreement with a separate analysis by CDC⁴).

As noted by *Ou et al.* and *DeBry et al.*, the interpretation of significance levels for bootstrap proportions is a matter of some debate^{5–7}. However, it is clear that bootstrap proportions (as usually formulated) considerably underestimate phylogenetic accuracy under most conditions⁵. We have shown that the probability of resolving the dental clade (the dentist plus patients A, B, C, E and G from the original study) is greater than 99% for various parsimony and distance methods, given the observed divergence and substitution frequencies⁸. Therefore, the individual bootstrap values shown in the table are also underestimates of phylogenetic accuracy, especially given the problem of multiple comparisons



across patients.

Our analyses agree with the conclusions of Ou *et al.*: there is significant support for phylogenetic trees that are consistent with the dental transmission hypothesis for patients A, B, C, E, G (and I), and significant evidence that patients D and F (as well as H and J) were infected from another source. These results are consistent with what is known about the existence of other risk factors for HIV among the 10 patients. DeBry *et al.* may turn out to be correct that regions other than the highly variable C2-V3 region will be even more informative about HIV transmission, but the C2-V3 data are nonetheless highly informative in this case.

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1. Ou, C.-Y. *et al.* *Science* **256**, 1165-1171 (1992).
2. Felsenstein, J. *Evolution* **39**, 783-791 (1985).
3. DeBry, R.W. *et al.* *Nature* **361**, 691 (1993).
4. CDC Mort. Morbid. Wkly Rep. **42**, 329-331, 337 (1993).
5. Hillis, D. M. & Bull, J. J. *Syst. Biol.* **42**, 182-192 (1993).
6. Felsenstein, J. & Kishino, H. *Syst. Biol.* **42**, 193-200 (1993).
7. Zharkikh, A. & Li, W.-H. *Molec. Biol. Evol.* **9**, 1119-1147 (1992).
8. Hillis, D. M. *et al.* *Science* (in the press).
9. Swofford, D. L. *PAUP 3.1: Phylogenetic Analysis Using Parsimony* (Illinois Biological Survey, Urbana, 1993).
10. Ciesielski, C. *et al.* *Ann. Intern. Med.* **116**, 798-805 (1992).

■ The HIV sequence data reported in ref. 3 are now publicly available. Genbank accession numbers are U06872 – U06919.

DNA from ancient Easter Islanders

SIR — Easter Island is one of the most remote inhabited places on Earth and there is considerable speculation about the origins of its prehistoric inhabitants. Early accounts of Easter Island are sketchy, but the population diverged from the pattern of Polynesian settlement and culture as a result of isolation and ecological degradation. The island was almost completely depopulated after European contact, and the present population of about 2,000 individuals is mostly Chilean. We report genetic evidence for the Polynesian origin of prehistoric Easter Islanders based on amplification and sequencing of mitochondrial DNA (mtDNA) from ancient human bones.

We analysed DNA from prehistoric skeletal material recovered from two discrete archaeological contexts, Ahu Tepeu on the west coast (1100–1680 AD), and Ahu Vinapu on the southern side of the island (1680–1868 AD). The bones were excavated by Thor Heyerdahl during his Norwegian Archaeological Expedition of 1955–56 (ref. 1) and kept in the Museo Nacional de Historia Natural, Santiago de

Chile. The relatedness of the individuals could not be inferred from the spatial distribution of graves as the skeletons were from secondary burials. We extracted DNA from bone samples of 12 adult individuals as previously described² and used it for polymerase chain reaction (PCR) amplification with primers specific for two anthropologically informative regions of the human mitochondrial genome. The first informative mtDNA fragment is in a small intergenic region between the cytochrome oxidase II (COII) and the lysyl transfer RNA genes that has two tandemly repeated copies of the 9-base-pair (bp) sequence CCCCTCTA (ref. 3). A length mutation consisting of a deletion of one of the two repeats is often found (5–40%) in present-day individuals of Asian origin, including Pacific islanders, and has reached fixation in some Polynesian islands⁴. All the prehistoric Easter Islanders were found to carry the 9-bp deletion, detected by electrophoresis of the PCR fragments through high-percentage agarose gels (see figure).

The second sequence analysed is a 228-bp fragment of the hypervariable control region of mtDNA, known to contain most of the sequence variability in the human mitochondrial genome. PCR products were sequenced directly after amplifica-



tion as described previously², and the sequences read between mtDNA bases 16,215 and 16,410 of the published human mtDNA reference sequence⁵. All the ancient Easter Island DNA samples had identical base substitutions, differing from the reference sequence at positions 16,217 (T→C), 16,247 (A→G) and 16,261 (C→T). One individual had an additional transition at position 16,271 (T→C) and two other individuals at position 16,292 (C→T).

The three substitutions at mtDNA positions 16,217, 16,247 and 16,261, in conjunction with the COII/trnA^{lys} 9-bp deletion, are typical of Polynesians. We previously observed these polymorphisms in most present-day Polynesians sampled (unpublished observations), and in prehistoric Polynesians from the Chatham Islands (near New Zealand) and Hawaii⁶. The survival of only one principal mitochondrial DNA lineage throughout remote Oceania is presumably due to population bottlenecks during the human colonization of the region. We have demonstrated that the prehistoric settlers of Easter Island derived from this identical lineage, characterized by the 9-bp deletion and the three base substitutions at mtDNA positions 16,217, 16,247 and 16,261. Our results confirm the Polynesian affinities of the original settlers and support the view that the unique culture that developed on the island resulted from the environmental conditions and several centuries of isolation.

Concerning the question of a possible settlement from South America, recent surveys of the hypervariable region of mtDNA of native Americans have shown much greater mtDNA heterogeneity in America than in Polynesia, reflecting multiple waves of migration and greater depth of prehistory, whereas the Polynesian type that we observed in Easter Island has not been detected^{7–10}. Although the 9-bp deletion exists throughout the Americas, Asia and the Pacific, often in association

1. Heyerdahl, T. & Ferdon, E. N. *Jr Monogr. Sch. Am. Res. and Kon-Tiki Mus.* No. 24, pt 2 (Santa Fe, 1965).
2. Hagelberg, E. & Clegg, J. B. *Proc. R. Soc. B* **244**, 45–50 (1991).
3. Wrischnick, L. A. *et al.* *Nucleic Acids Res.* **15**, 529–542 (1987).
4. Hertzberg, M., Mickelson, K. P. N., Serjeantson, S. W., Prior, J. F. & Trent, R. J. *Am. J. Hum. Genet.* **44**, 504–510 (1989).
5. Anderson, S. *et al.* *Nature* **290**, 457–465 (1981).
6. Hagelberg, E. & Clegg, J. B. *Proc. R. Soc. B* **252**, 163–170 (1993).
7. Ward, R. H., Frazier, B. L., Dew-Jager, K. & Pääbo, S. *Proc. natn. Acad. Sci. U.S.A.* **88**, 8720–8724 (1991).
8. Ginther, C. *et al.* in *DNA Fingerprinting: State of the Science* (eds Pena, S. D. J., Chakraborty, R. & Jeffreys, A. J.) 211–219 (Birkhäuser, Basel, 1993).
9. Horai, H. *et al.* *Molec. Biol. Evol.* **10**, 23–47 (1993).
10. Torroni, A. *et al.* *Am. J. Hum. Genet.* **53**, 563–590 (1993).

with the T→C transition in position 16,217, this simply supports the view of the shared common origin of the ancestral settlers of Oceania and the New World. The absence of the other two Polynesian substitutions (mtDNA sites 16,247 and 16,261) in the New World points to a lack of significant contact between Polynesia and the Americas.

Our findings confirm the usefulness of molecular-genetic analysis of skeletal remains for addressing questions in prehistory and evolution. Although there are considerable technical problems in analysing ancient DNA, including DNA damage and artefacts caused by contamination, as well as the anecdotal nature of much of the data, these difficulties are not insuperable. The bones used in this study were stored for almost 40 years following their excavation by the Heyerdahl team and studied by several groups of physical anthropologists without special precautions to avoid contamination. Nevertheless, unambiguous genetic information could be recovered from them.

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Hypothetical SisterKiller

SIR — It was premature of Hurst in his News and Views article¹ to accept Haig's claim² that a hypothetical meiotic drive element, SisterKiller, can lead to evolution from one-step to multi-step meiosis. The basis of Haig's claim is that a SisterKiller allele that causes a gamete to kill its sister gamete can invade and go to fixation in a population using one-step meiosis, whereas in a population using multi-step meiosis, SisterKiller cannot invade. Hurst concludes that SisterKiller could have caused the evolution of multi-step meiosis. The conditions under which this is true are more restrictive than Haig suggests.

Some simple algebraic models clarify the issues. Let p be the frequency of SisterKiller in a haploid population using one-step meiosis to produce two gametes.

By hypothesis, a homozygote and a heterozygote for SisterKiller will produce only a single gamete, in both cases containing the SisterKiller allele. Assuming random mating, the recurrence equation for the frequency of the SisterKiller allele is

$$p' = \frac{p(2-p)}{2(1-p) + p^2}$$

There are two equilibria, $p = 1$ and $p = 0$. The equilibrium at $p = 0$ is unstable, indicating that SisterKiller can invade. Once present, SisterKiller will increase in frequency until fixed. However, when rare the change in frequency per generation is extremely low. Letting

$$p = \frac{1}{N}$$

where N is the haploid population size, yields

$$p' = \frac{1}{N} + \frac{1}{2N^2} +$$

indicating that SisterKiller is nearly neutral when rare. Still, a SisterKiller allele may eventually drift up to the point where its advantage becomes significant and so can invade.

Now consider the same model as above but with non-random mating measured by the inbreeding coefficient F . The recurrence equation is readily modified³ to yield

$$p' = \frac{p(2-F-(1-F)p)}{2(1-p) + Fp + (1-F)p^2}$$

which has three equilibria, $p = 1$, $p = 0$ and

$$p = \frac{F}{1-F}$$

When the third, intermediate, equilibrium exists it is unstable and the equilibrium at $p = 0$ becomes stable. Any amount of inbreeding ($F > 0$) brings in the intermediate equilibrium. SisterKiller when rare is then no longer nearly neutral but instead is deleterious. The high inbreeding that Hurst says would be required to prevent the invasion of SisterKiller is not necessary. SisterKiller, as defined by Haig, is unlikely to invade any large population using one-step meiosis.

It is possible to rescue SisterKiller by introducing a direct benefit for attacking (or consuming) the sister gamete. Haig

suggests this variant but does not pursue it. Let b be the direct benefit of SisterKiller's attack, such that the relative viability of SisterKiller gametes produced by SisterKiller homozygotes and heterozygotes is $1 + b$. We then find that the resulting recurrence equation has three equilibria, $p = 1$, $p = 0$, and

$$p = \frac{F + b(F-2)}{1-F-b(1-F)}$$

When this third equilibrium is between 0 and 1, the equilibrium at $p = 0$ is rendered stable as above and SisterKiller cannot invade. The unstable intermediate equilibrium coalesces into the zero equilibrium which becomes unstable when

$$b \geq \frac{F}{2-F}$$

SisterKiller must derive benefit exceeding half the inbreeding coefficient to invade a population using one-step meiosis.

Finally, showing that a multi-step meiosis, when fixed, can prevent invasion of SisterKiller, does not support SisterKiller's role in the evolution of multi-step meiosis. Evaluating the impact of meiotic-drive elements on the evolution of multi-step meiosis from one-step meiosis via individual selection requires dynamic models describing the change in frequency of alleles controlling the method of meiosis, similar to those for the evolution of resistance of meiotic-drive elements through recombination⁴, modifiers⁵ and inter-deme selection⁶. These models have yet to be constructed and analysed, so SisterKiller's impact on the evolution of multi-step meiosis remains even more hypothetical than does SisterKiller itself.

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1. Hurst, L. *Nature* **365**, 206–207 (1993).
2. Haig, D. J. *theor. Biol.* **163**, 15–31 (1993).
3. Wright, S. *Evolution and the Genetics of Populations II* (Univ. Chicago Press, 1969).
4. Haig, D. & Grafen, A. *J. theor. Biol.* **153**, 531–558 (1991).
5. Prout, T., Bundgaard, J. & Bryant, S. *Theor. Populat. Biol.* **4**, 446–465 (1973).
6. Leigh, E. G. Jr *Proc. natn. Acad. Sci. U.S.A.* **74**, 4542–4546 (1977).

HAIG REPLIES — The argument that SisterKillers favour two-step meiosis was always intended to apply to the case of direct local benefits. Butcher and Deng's own results show that, in the absence of inbreeding, any local benefit whatsoever ($b > 0$) allows the invasion of a SisterKiller. Such a condition for successful sororicide does not seem prohibitive.

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Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. Priority will be given to letters of fewer than 500 words and five references.

Consumable evils

Thomas H. Jukes

The FDA Follies: An Alarming Look at Our Food and Drugs in the 1980s. By Herbert Burkholz. BasicBooks: 1994. Pp. 228. \$23.

The general theme of *FDA Follies* is that "by the end of the 1970s, the agency [US Food and Drug Administration, or FDA] enjoyed a worldwide respect as the finest regulatory body of its kind" (p. 11), but in the 1980s, the author claims, the FDA went to pot because Presidents Reagan and Bush encouraged cooperation, rather than an adversarial relationship, between the FDA and industry. In the final chapter, the author sees faint rays of hope: Reagan and Bush are gone. Can the FDA's commissioner David Kessler make the agency work? In the intervening pages, the story of the 1980s is described.

The sudden assignment of increased regulatory powers to the FDA typically follows a catastrophe. In 1937, Massengill sold an untested solution of sulphanilamide in ethylene glycol, which promptly killed 107 people. As a result, the Food, Drug and Cosmetic Act was passed in 1938. The second catastrophe, that of thalidomide, a tranquillizer, was kept out of the United States by the FDA, but its occurrence elsewhere led to strong amendments to the act.

On average, it takes 12 years and \$231 million to develop a new medicine "and only one in five makes it to final approval". New-drug applications "typically run to 100,000 pages or more". New drugs are usually protected by patents and sold under brand names. When patents expire, it is possible for generic drugs to compete with brand names. But approval for the sale of a generic drug is in the hands of the FDA and the agency has to resolve the scramble for new-drug applications by competing companies. Some of the companies lack manufacturing expertise. In Chapter 2, the author describes how the scramble may lead to payoffs for FDA employees; an investigation in 1988 led to the conviction of 42 people and 10 companies for fraud or corruption.

The author points out that the FDA is concerned with safety and efficiency, and not with pricing. Yet the price of drugs has gone steadily up, increasing by nearly three times the rate of inflation between 1986 and 1992.

In 1959, thalidomide was manufactured by the German company Chemie Grunenthal, and was sold in many countries outside the United States. A US company, Richardson-Merrell, applied to the FDA to market the drug in the United States. The application was channelled to Frances Kelsey, a well-qualified pharmacologist who "fought a dogged defensive

battle". Neither Chemie Grunenthal nor Richardson-Merrell could or would answer her questions. She refused to grant approval.

Gradually the news filtered through from Europe. Thalidomide given to women during early pregnancy produced phocomelia in their infants. In this dreadful affliction, "some had no arms, just flippers . . . others had limbless trunks with toes extending from their hips . . . in the end, more than eight thousand children in 46 countries were affected".

When the thalidomide story was revealed, and the pressure from industry to approve thalidomide became known, "the legislative clout of the drug companies began to dissipate rapidly". The Kefauver-Harris Drug Amendments to the federal Food and Drug Act was passed, requiring drugs to be proved safe and effective. Curiously, Burkholz does not commend the FDA for saving the United States from thalidomide.

The author describes the vociferous pressure exerted by AIDS activists for the

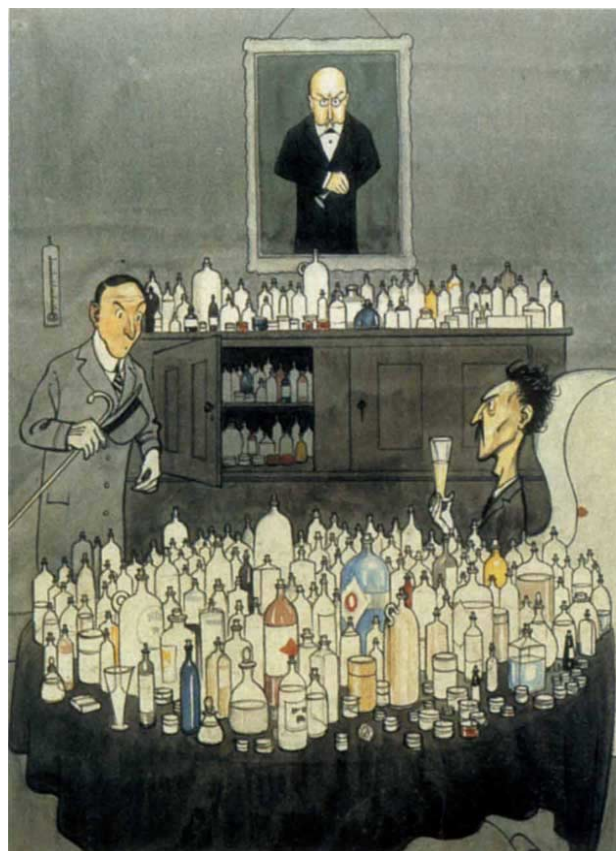
release of new drugs for AIDS treatment. As a result of this pressure, by the end of the 1980s, the FDA's drug-approval process "had been radically altered". But are the new anti-AIDS drugs any good? Are we better off with quick release or with Dr Kelsey?

The Dalkon shield is an intrauterine device that stops pregnancy by inhibiting attachment of the fertilized ovum. It has prongs that stick into the uterine wall and a wick that allows pathogenic bacteria to enter the uterus. Marketed in 1970 without hindrance from the FDA, the shield led to septic abortions and deaths. No action was taken by the FDA and, by 1980, the manufacturer used the federal bankruptcy law to halt 6,000 lawsuits and to limit the company's liability to \$2.4 billion for 200,000 women.

Another device that led to disasters was a heart valve approved by the FDA in 1979. It broke up within patients with "a deadly regularity" of about 7 of every 10,000 recipients each year. Eventually, its manufacturers withdrew it from the market in 1986 and settled a class-action suit by paying several hundred million dollars.

The latest controversial device is still in the headlines: silicone-gel breast implants. Burkholz says that the FDA failed to ask basic questions about these implants. Last month, there was news that breast-implant companies have joined in a

"A MEMBER of the Society of Hopeless Hypochondriacs" by H. M. Bateman, 1915. Medical advertising has often been criticized for encouraging more gullible members of the public to imagine themselves suffering from all sorts of specious illnesses. This watercolour is currently on display in "Pills and Profits: The Selling of Medicines Since 1870", an exhibition that opened last week at the Wellcome Institute for the History of Medicine in London. Ranging from a Rembrandt etching to modern promotional calendars and television clips, the exhibition, which closes on 19 August, looks at changes in medical commercial practice and styles.



settlement of \$4.7 billion to be shared by thousands of women who have blamed the implants for various illnesses. I wonder whether breast implants and Dalkon shields might both have been rejected had women been in charge.

Burkholz gives a lively account of the "Chilean Grape Scare of 1989", in which Chile's economy was severely damaged by the announcement that two (yes, two) Chilean grapes in a shipment that landed in Philadelphia were found to be contaminated with cyanide. He does not reveal that the cyanide content was 3 micrograms per grape. As a result of the 'scare', the FDA issued a ban on Chilean fruit on 14 March 1989. The key information, not provided by Burkholz, is that many foods contain low levels of cyanide, present in cyanogenic glycosides such as linamarin. A 1-gram lima bean would typically contain 100 micrograms of cyanide in linamarin, from which cyanide is set free by common enzymes. The two grapes contained a total of 6 micrograms. No more tainted grapes were found in the next 12,000 crates of Chilean grapes inspected by the FDA. No wonder the Chilean government demanded monetary compensation.

Burkholz does not mention the active programme of the FDA in court actions against health fraud. And a final point worth noting: the FDA has kept the "Sensor Pad" off the market for the past 9 years (*Wall Street Journal*, 12 April 1994). This is a non-invasive device for aiding women to examine their own breasts. It is approved and widely used in other countries. *Plus ça change, plus c'est la même chose.* □

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New Journals issue

This year, *Nature's* annual New Journals review supplement will appear in the issue of 29 September. Publishers and learned societies are invited to submit journals for review, taking note of the following criteria:

- Journals that first appeared during or after June 1992 and issued at least four separate numbers by the end of April 1994 will be considered.
- Journals covering any aspect of science are eligible, although those dealing with clinical medicine, engineering and pure mathematics are excluded, as are publications of abstracts.
- Frequency of publication must be at least three times a year. The main language used must be English. Translation journals in English are, of course, eligible.
- Deadline for submission is the end of May.

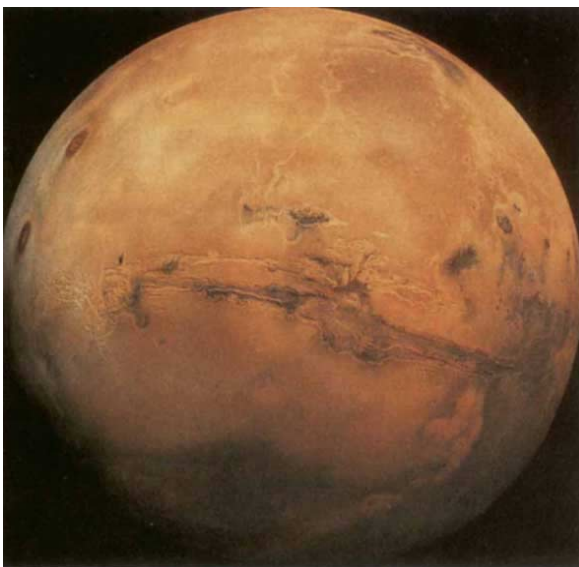
When submitting journals for review, please send at least four different issues (the first, the most recent and any two others) of each title, together with full details of subscription rates (personal and institutional) and frequency of publication to: Peter Tallack, *Nature*, 4 Little Essex Street, London WC2R 3LF, UK. For further information please telephone Peter Tallack on +44 71-836-6633 (international), extension 2414.

The Martian chronicles

Joel Gwinn

The Explorers of Mars Hill. By William Lowell Putnam. *Phoenix: 1994. Pp. 289. \$30.*

THE second half of the nineteenth century witnessed a growing wave of interest in planetary astronomy. In the United States, many universities and other institutions acquired refracting telescopes with large objective lenses, instruments that required correspondingly large financial support. Lick Observatory and Yerkes Observatory were both funded by wealthy individuals who aspired to construct the 'world's largest telescope'.



A Viking orbiter mosaic image of the Valles Marineris region of Mars. Taken from *Images of the Cosmos* by B. W. Jones, R. J. A. Lambourne and D. A. Rothery (Hodder & Stoughton, £12.99 (pbk)), a rich photographic portrait of the Universe. Each item is selected by experts from the Open University in the UK.

Lowell Observatory, however, was built by a wealthy amateur astronomer who had already selected a planet for study.

Percival Lowell (1855–1916), scion of a patrician Boston family, became interested in astronomy while still a teenager. At Harvard University, he excelled in mathematics and graduated with honours, choosing a topic in astronomy for his thesis. He became captivated by the reported sightings by Schiaparelli, the distinguished Italian astronomer, of "canali" (mistranslated as "canals"), peculiar markings on the Martian surface.

In 1894, Mars was to be in a particularly favourable position for observation by astronomers. Lowell resolved to continue the Martian studies of Schiaparelli. Enlisting the help of W. H. Pickering, a professor at Harvard, and A. E. Douglass, a recent astronomy graduate, Lowell began

a search for a suitable location for a new observatory. By the end of May 1894, using borrowed telescopes, the three astronomers were making observations of Mars at the new observatory site at Flagstaff in central Arizona, a frontier town that eagerly welcomed the new institution, helping with construction of roads and facilities and providing grants of land.

Thus started the first century of astronomical observation and research at Lowell Observatory. The institution that began as Lowell's own observatory is still today the world's largest privately owned and operated observatory. His great energy and financial resources made it possible for the centre to exploit the latest technology.

Lowell's reports on the controversial Martian canals, and his theory of a Martian civilization struggling to survive on a

dwindling supply of water on the planet, met much opposition from the scientific community, and inevitably put the observatory in a slightly embarrassing position. In 1901, Douglass, by this time "Assistant in Charge" of the observatory, wrote an earnest and heartfelt letter to William Lowell Putnam II, who was chief administrator during an extended absence of Percival Lowell, urging Putnam to use his influence to persuade Lowell to take a more scientific approach, or to confine his activity to popular writing. Lowell's lectures and writings, such as *Mars* and *Mars and its Canals*, continued to be prominent in the public view, but Douglass had to find a new job — he moved on to a long and distinguished career at

the University of Arizona — and Lowell returned to rebuild the scientific staff of his observatory.

One new member of the staff was Vesto M. Slipher. Using a spectrograph provided for the investigation of "spiral nebulae", thought by Lowell to be solar systems in an early formative stage, Slipher was the first to obtain, in spectrograms of these spirals, absorption lines that revealed large Doppler shifts implying large radial velocities. More than ten years later, Edwin Hubble, working at Mount Wilson, was to complete the proof that these 'spiral nebulae' are, in reality, galaxies outside the Milky Way, receding from it and from each other in an expanding Universe. Slipher's landmark discovery is just one of many achievements at Lowell Observatory.

The work most closely associated with

the observatory is the discovery of the ninth planet, Pluto, early in 1930. Lowell had made extensive calculations based on observed discrepancies in the orbit of Uranus to predict the position of a "Planet X" beyond Neptune, which itself had been found through analysis of the gravitational effect of Neptune on Uranus.

Partly because the newly discovered planet turned out to be of much lower mass and brightness than predicted, some controversy has existed about whether the discovery may be attributed to the theoretical predictions as opposed to the long, thorough search.

In this centennial volume, William Putnam proudly shares the excitement of these achievements. He includes contributions by others involved in the 100-year history of the observatory, tells the

personal stories of the people involved in the life and work of the observatory and captures the essential human experiences of both failure and success. All the anecdotes are entertaining, although some are only loosely connected with the astronomical work. According to Putnam, some of the tales are true.

As the current designated successor trustee of Lowell Observatory, Putnam has been able to include many interesting pictures and stories about Lowell and his observatory. This handsomely produced book makes fascinating reading for anyone interested in the astronomical frontiers of the past hundred years. □

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triguing but marred by hearsay) than the newly released Einstein correspondence. He deals with the biography's explosive thesis — that Mileva deserved joint credit for special relativity — but does not dispose of it as convincingly as John Stachel of Boston University, who is not cited in the book's copious references.

Less provocative than Truhović, though more interesting, is the correspondence in volumes one and five of the collected papers. Pais does little with this rich material to recreate mood or provide insight. We get the occasional whiff from Einstein's own words ("We remain students as long as we live and don't give a shit for the world") but the chapter is flat.

Pais trots out quotations and facts but there is little attempt to extrapolate what was going on in Einstein's head. It is rather like the scientist who is happy to put data points on a plot but unwilling to draw a line through them. (For a taste of how it can be done, see the perceptive introduction of Jürgen Renn and Robert Schulmann's *Albert Einstein and Mileva Marić: The Love Letters*, Princeton University Press, 1992; for a review see *Nature* 360, 377; 1992).

Pais litters his book with the scholarly equivalent of a nod and a wink; he tells us he has seen Einstein's divorce decree but does not quote it. He tells us that Einstein became intimate with his cousin Elsa in Berlin but we see nothing of the clandestine letters he wrote to her. Einstein also had an affair with a young woman in the 1920s but Pais never cites their correspondence or identifies her (believed to be his secretary, Betty Neumann), even though this was one of the most significant of Einstein's affairs. At one point, Pais portentously announces he will break a "promise of silence" — and the reader learns that Einstein sold a manuscript to pay for his home in Princeton.

You may ask why we need to know more about Einstein's failings, notably his shaky relationship with his wives and sons. It is more than the Big Question. Poor Mileva is usually portrayed as a morose harridan who cramped Einstein's style. You can almost hear the traditional biographers shout "good riddance" when in 1914 she left a tearful Einstein in Berlin and took their sons to Zurich. You can almost hear the hurrah when his cousin Elsa supposedly enters the story in 1917 to tend him on his sick bed and then fall in love with him.

This is unfair to Mileva. There is evidence of Einstein's wandering eye during their marriage in 1909, when an old flame resumed contact with him, and in 1912, when he struck up an intimate correspondence with Elsa in Berlin. In the letters that Pais overlooks, Einstein curses Mileva as his "cross", and "the sourest sourpot there has ever been". No wonder Mileva found the family move to Berlin —

Lotte Jacobi

Albert and his private lives

Roger Highfield

Einstein Lived Here. By Abraham Pais. Oxford University Press: 1994. Pp. 282. £14.95, \$25.

AMONG my collection of books on Einstein, there is a dog-eared copy of Abraham Pais's scientific biography *Subtle is the Lord*. Its poor condition pays tribute to the value of this brilliantly researched book, one that stood out from the crop published around the centenary of Einstein's birth.

In recent years, a remarkable amount of additional correspondence has become available to scholars, thanks to the diligent efforts of the Einstein Papers Project at Boston University and the death of three individuals close to Einstein who harried biographers and used legal action to prevent anything too revealing from being published, perhaps even 'lost' letters.

The newly released letters have stimulated a re-evaluation of the personality of the young Einstein, revealed he had an illegitimate daughter and stirred debate about the origins of his science, including a highly publicized row over the role of his first wife, Mileva, in the development of special relativity. Rather than update *Subtle is the Lord*, Pais has decided to draw on this and other material, including decades of newspaper cuttings, to provide "essential information about Einstein the human being".

This is a good idea. After all, Pais tells us that he knew Einstein well. And it helps that Pais avoids the main defect of *Subtle is the Lord*: blending the man and his mathematics produced an inelegant structure, consisting of slabs of science stuck together with biographical mortar.

Einstein Lived Here is aimed at non-

physicists. This is just the readership that would be left hungry for insights into Einstein's character if they had read only the traditional hagiographies, which count compassion, wisdom and good humour alone as his 'personal side'. Most people also want to find out whether Einstein had a darker aspect to his personality. In

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Wolf in sheep's clothing? — "we need to know about Einstein's failings".

short, they want to know what Einstein was like.

I sought the answer to this, the Big Question, in *Einstein Lived Here*. The first chapter on Einstein's family is so staccato that it reads like a curriculum vitae. It has a puzzling opening, suggesting that Pais was more inspired to put pen to paper by the 1969 biography of Mileva Marić by Desanka Truhović-Gjurić (in-

and Einstein's mistress — intolerable.

These personal details illuminate fundamental issues: the link between Einstein's creativity and his emotional state, and why his marriage fell apart and his eldest son bore him a grudge. Even though there is no-one to hurt by such revelations, Pais decides that the reader should be spared them. Like Carl Seelig, he had greater access to Einstein's secrets than most other biographers, but perhaps he also felt so much a part of the family that he kept them to himself.

It is also telling that Pais pays special tribute to Helen Dukas, the woman who spent 27 years at Einstein's side while he faced threats ranging from oppressive media attention to the Nazis and the McCarthy investigation of supposed communists. She was liable to attack as "dung" any biographers who dared to shed light on Einstein's personal life.

The remaining chapters demonstrate that a collection of essays does not necessarily make a book. There is no fundamental unifying theme, although Pais is more comfortable moving away from Einstein's family life to his crank mail, his

Nobel prize and his relationship with religion, philosophy and the media. Pais diligently collates a wide range of information, uncovers some striking Einstein quotations that are as true today as 60 years ago, and crisply summarizes the various themes. I wish, however, there had been tougher editing: the main essay of the book, on Einstein and the press, reads too much like notes, some parts are dull (try, for instance, the section on: "The return home via three weeks in Spain") and the analysis too brief.

But much of my criticism reflects disappointment that Pais has not fulfilled our high expectations of the author of *Subtle is the Lord*. Some biographers are still attempting to research Einstein's story without coming within a mile of an archive, scholar or Einstein relative. The pot-boilers that result are totally outclassed by *Einstein Lived Here*. Scholarly, thoughtful and entertaining in parts — but quite unable to answer the Big Question. □

Roger Highfield is science editor at The Daily Telegraph, and author with Paul Carter of *The Private Lives of Albert Einstein* (Faber, 1993/St Martin's, 1994).

Family values in signal transduction

Alan Hall

The G-Protein Linked Receptor FactsBook. By Steve Watson and Steve Arkin-stall. Academic Press: 1994. Pp. 427. £19.50, \$29.95.

A RECENT article in *Nature* reported that only one in three genes emerging from the project to map the genome of the nematode worm *Caenorhabditis elegans* was similar to a previously known gene. This contrasts strikingly with the experience of those of us in the signal-transduction field where it seems almost inevitable that each new protein identified is either just one member of a larger family or at least contains a domain found in numerous other proteins. It's a struggle to understand much of this complexity and apparent functional redundancy, let alone remember all the names. However, all is not despair, except perhaps for those doing gene knock-outs, and there is some satisfaction to be gained from comparing families and superfamilies of related proteins, as it reveals the essential conservatism of evolution: making the most of a good idea.

Seven transmembrane receptors are a classic example of a 'good idea'; estimated to number in the thousands (well over a hundred are described in this book), their overall structures are highly conserved and they each generate an intracellular signal by first coupling to a GTP-binding

protein. Even prokaryotes realized that the GTP-binding protein was a simple and effective way of controlling biochemical processes and an impressive array of regulatory proteins has evolved in eukaryotic cells based on this principle. The heterotrimeric G-protein family specifically couples seven transmembrane receptors to second messenger enzymes and more than 20 varieties have been found in mammalian cells. This *FactsBook* assembles the sequence, specificity and functional properties of the known seven transmembrane receptors and their associated G proteins. In addition, although they are not related structurally or functionally to each other, similar information is presented for the downstream second messenger-generating enzymes.

Compilations such as this are becoming increasingly necessary to bring order and accessibility to the vast amount of new information generated on protein function. This *FactsBook* is extremely well presented and referenced and is user-friendly. It will be an essential source of reference for anyone actively involved in signal transduction research. □

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New in paperback

Space, Time and Man: A Prehistorian's View by Grahame Clark. Canto (Cambridge University Press), £5.95, \$9.95. "A deep appreciation of our historical roots, seen in a very large frame" (Owen Gingerich, *Nature* 358, 381; 1992).

Quantum Physics: Illusion or Reality? by Alastair Rae. Canto (Cambridge University Press), £4.95, \$8.95. A clear introductory exploration of the theories on offer.

A Dancing Matrix: How Science Confronts Emerging Viruses by Robin Marantz Henig. Vintage, \$12. Now updated. The first edition of this book was described by Beverley Griffin as "vital and stimulating. . . once picked up, it is difficult to put down" (*Nature* 364, 201; 1993).

The Language of the Genes: Biology, History and the Evolutionary Future. HarperCollins, £6.99. "This intelligent and informative book deserves to be read widely, since it provides a vivid demonstration of what a remarkable and fragile product of evolution our species is" (C. Wills, *Nature* 364, 113; 1993).

The Planets: Portraits of New Worlds by Nigel Henbest. Penguin, £15, \$17.95. "Lavishly illustrated in colour and the pictures are of the highest quality. . . an excellent, up-to-date, general introduction to space-age planetary astronomy" (David Hughes, *Nature* 359, 684; 1992).

The Diversity of Life by Edward O. Wilson. Penguin, £7.99. "A deft and thoroughly successful mixture of information and prophecy. . . a great and fit work destined to survive and thrive well into the next millennium. . . Wonderful writing" (Stephen Jay Gould, *Nature* 361, 311; 1993).

Eight Little Piggies: Reflections in Natural History by Stephen Jay Gould. Norton/Penguin, \$10.95. "A lovely mixture of bizarre facts, nice arguments, clever insights into the workings of evolution and a quality of writing that can make your skin prickle. . . Gould has given us a feast" (Bryan Clarke, *Nature* 362, 656; 1993).

Who's who in science

Two newly revised scientific bibliographical reference guides have just been published. **Biographical Encyclopedia of Scientists** edited by J. Daintith, S. Mitchell, E. Toothill and D. Gjertsen (2nd edn; 2 vols; pp. 1,075; 15 contributors) contains a main biography section of some 2,000 entries, each of about 300 words; shorter sections listing chronologies of scientific discoveries, scientific institutions, and influential books and papers; and name and subject indexes. Institute of Physics Publishing, £120, \$190. **Collins Biographical Dictionary of Scientists** edited by Trevor Williams (4th edn; pp. 602; 60 contributors) provides generally longer accounts of more than 1,300 individuals, each with bibliographical details for further reading. It ends with chronological listings of Nobel laureates and anniversaries, an appendix of names and a subject index. HarperCollins, £25.

Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice

David Kägi, Birgit Ledermann*, Kurt Bürki*, Peter Seiler, Bernhard Odermatt, Kristin J. Olsen†, Eckhard R. Podack†, Rolf M. Zinkernagel & Hans Hengartner

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Perforin-deficient mice have been generated by homologous recombination to determine whether the effects of CD8⁺ cytolytic T cells and natural killer cells are mediated by pore formation involving perforin. These mice are viable and fertile and have normal numbers of CD8⁺ T cells and natural killer cells which do not lyse virus-infected or allogeneic fibroblasts or natural killer target cells *in vitro*. The mice fail to clear lymphocytic choriomeningitis virus and they eliminate fibrosarcoma tumour cells with reduced efficiency. Perforin is therefore a key effector molecule for T-cell- and natural killer-cell-mediated cytotoxicity.

THERE is ample evidence that CD8⁺ cytotoxic T cells and natural killer (NK) cells are involved in the elimination of some viruses, in graft rejection¹, in antitumour immune response, in immunopathology and in some autoimmune diseases. Although cell-contact-dependent cytolytic activity is the functional hallmark of CD8⁺ and NK cells *in vitro*, it is debated whether lytic activity or secretion of soluble factors, or a combination of both, mediate their activity *in vivo*²⁻⁴. To account for lymphocyte-mediated cytotoxicity, several mechanisms have been suggested, among them the granule exocytosis model, programmed cell death triggered through Fas or other target cell intrinsic pathways^{5,6}, and the action of serine esterases⁷, tumour-necrosis factors and analogues. Based on morphological and biochemical evidence⁸⁻¹⁰, the granule exocytosis model of lymphocyte-mediated cytotoxicity proposes that cytolytic lymphocytes directionally release the content of cytolytic granules upon specific recognition and conjugate formation with a target cell^{11,16}. Because of its ability to form pores upon polymerization¹², perforin is a plausible granular molecule to be responsible for cell lysis^{13,14}. This functional similarity between perforin and complement C9 is also reflected in the considerable homology between their amino-acid sequences^{15,16}.

Perforin is expressed in both cytotoxic T lymphocytes (CTLs) and NK cells¹⁶⁻¹⁹ and has been histologically detected in situations in which cytolytic lymphocytes are presumably involved, such as during graft rejection, antiviral immune response and immunopathology²⁰⁻²². To test the role of perforin directly we generated perforin-deficient mice by homologous recombination in embryonic stem (ES) cells. These mice are viable and fertile and have normal numbers of CD8⁺ and NK-marker-positive cells. However, antiviral and transplantation antigen-specific cytotoxic T-cell activity against fibroblast target cells is absent, as is NK activity measured *in vitro*. Perforin-deficient mice are not able to clear infection with lymphocytic choriomeningitis virus and have a reduced capacity to control the growth of syngeneic fibrosarcoma tumour cells. These results demonstrate that pore formation by perforin is the main mechanism of cytotoxicity mediated by CTLs and NK cells *in vitro* and a crucial effector mechanism *in vivo*.

Generation of perforin-deficient mice

The murine perforin gene contains one untranslated and two translated exons (Fig. 1b)^{23,24}. To target one allele of perforin in ES cells²⁵, we made a replacement vector containing a 3.3-kilobase (kb) genomic fragment which included DNA from exon 3 and the preceding intron (Fig. 1a). A *neo* cassette (for neomycin resistance) was inserted in parallel orientation into the *Bst*III site of perforin exon 3. Upon electroporation into BL/6-III ES cells²⁶, six homologous recombinant ES cell clones were obtained at a frequency of 1 in 30 G418-resistant cells. Interestingly, an identical construct containing the *neo* cassette in reverse orientation yielded a frequency of only 1 in 200.

Homologous single-copy integration in the ES clones was confirmed by Southern blot analysis using a flanking probe (A) and two probes (B, C) specific for the construct (Fig. 1c). One targeted ES cell clone gave rise to germ-line transmitting chimaeras upon injection into BALB/c blastocysts and subsequent implantation into foster mothers. The chimaeras were bred with C57BL/6 mice, and heterozygous perforin +/0 offspring were identified. Perforin 0/0 animals were obtained by interbreeding heterozygous animals (Fig. 1d).

Because the BL/6-III ES cell line is of C57BL/6 origin, perforin-deficient mice basically represent a mutant C57BL/6 strain. Young mice proved to be susceptible to mouse hepatitis virus, but after reaching a critical age of about 8 weeks, they survived well under conventional animal housing conditions.

Characterization of perforin-deficient mice

The *neo* cassette was inserted into the perforin gene without deletion of coding sequence. To demonstrate that no fully functional perforin messenger RNA was produced by splicing out of the *neo* cassette at the mRNA level, spleen mRNA from animals stimulated by infection with lymphocytic choriomeningitis virus (LCMV) was analysed by reverse transcription and polymerase chain reaction (RT-PCR) (Fig. 1e). Bands of similar intensity from normal and perforin 0/0 mice were obtained with exon 1/2 specific primer pair a/b (Fig. 1e), exon 2/3-specific primer pair c/d, and a β -actin-specific primer pair, indicating that similar amounts of mRNA were retrotranscribed and that transcrip-

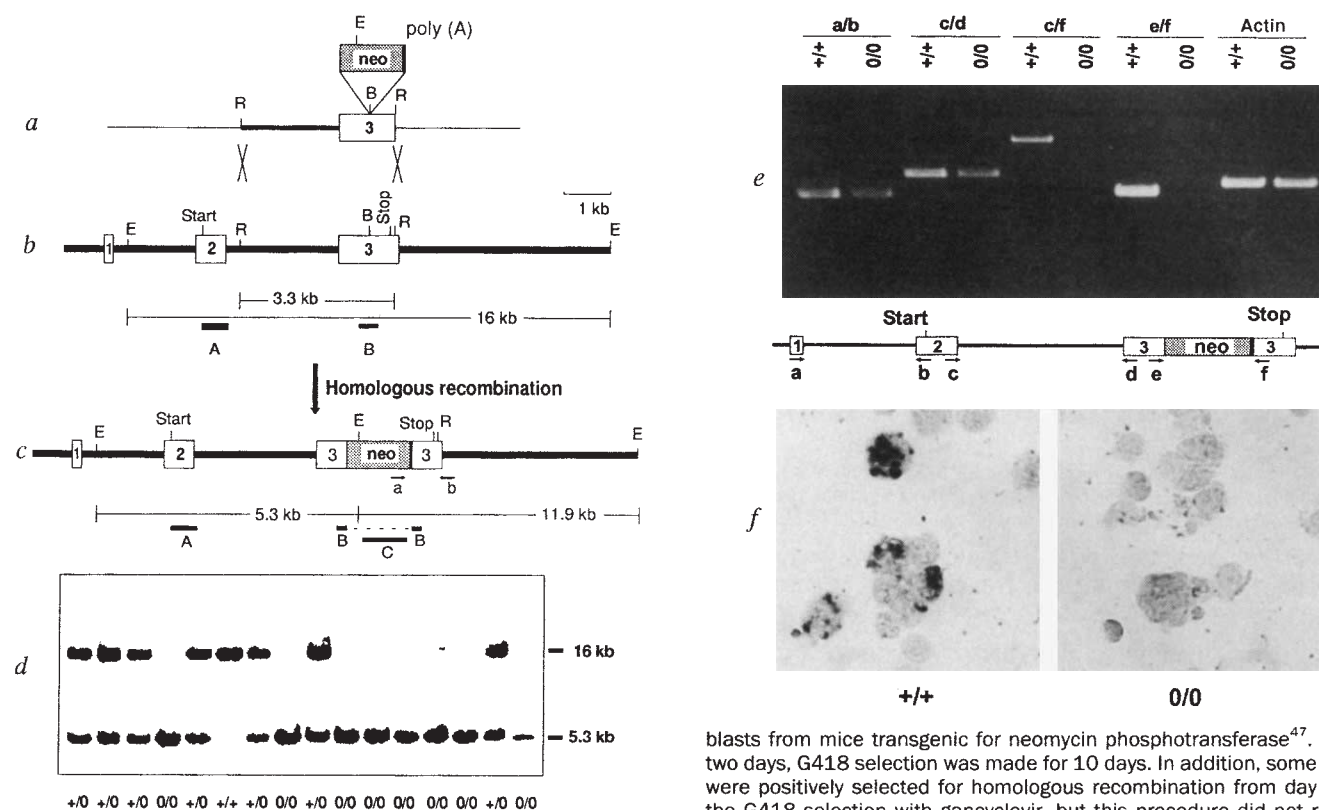


FIG. 1 Disruption of the perforin gene in mouse embryonic stem (ES) cells by homologous recombination. **a**, The targeting construct pCI.HK2 consisted of a 3.3-kb *EcoRV* fragment from perforin exon 3 and from a part of the preceding intron, interrupted by a *neo* cassette. *EcoRI* (E), *EcoRV* (R) and *BstEII* (B) sites are indicated. **b**, Map of the murine perforin gene and the hypothetical crossover events. *EcoRI* digestion and Southern blot analysis with an exon 2 probe (A) yield a 16-kb band for the unmutated locus. **c**, The disrupted allele exhibited an additional *EcoRI* site, which gives rise to a diagnostic 5.3-kb *EcoRI* fragment hybridizing with a flanking probe (A). The mutation was detected by PCR using oligonucleotides a and b. **d**, Southern blot analysis of a litter from a heterozygous $+ / 0 \times + / 0$ breeding. Tail DNA was digested with *EcoRI* and hybridized to flanking probe A. The 16-kb wild-type and 5.3-kb mutant fragment are indicated. **e**, Analysis of perforin mRNA. cDNA from spleens of LCMV-infected normal and 0/0 mice were analysed by PCR amplification with the indicated perforin-specific and control β -actin specific primers. The absence of PCR signals from 0/0 cDNA with primer pairs c/f and e/f shows that the *neo* cassette is not deleted by splicing. **f**, Perforin expression of perforin 0/0 mice. Activated spleen cells from normal and perforin 0/0 mice were stained with an anti-perforin antibody⁴⁵.

METHODS. **a**, The targeting construct was obtained by inserting a 1.2-kb *neo* cassette into the *BstEII* site of a 3.3-kb fragment from perforin exon 3 and the preceding intron. The neomycin phosphotransferase gene was driven by a herpes simplex virus thymidine kinase promoter engineered for expression in ES cells and contained a polyadenylation signal²⁵. In addition, the construct included a herpes simplex thymidine kinase gene from plasmid pCI19.MCI-TK⁴⁶. The linearized construct with thymidine kinase, neomycin resistance and perforin gene oriented in the same direction was used to electroporate male BL/6-III embryonic stem (ES) cells originally generated from the C57BL/6 mouse strain²⁶. After electroporation, ES cells were grown on embryonic fibro-

blasts from mice transgenic for neomycin phosphotransferase⁴⁷. After two days, G418 selection was made for 10 days. In addition, some cells were positively selected for homologous recombination from day 7 of the G418 selection with gancyclovir, but this procedure did not result in more targeted events. When colonies were grown to ~50 cells on day 10 or 11 of G418 selection, a crude DNA preparation from half of each colony was analysed by PCR amplification with primers a (5'-TGG GCT TCA GTG GCG TCT TG-3') and b (5'-TGT GTG GCG TCT CTC ATT AG-3') to detect homologous recombination. PCR-positive clones were grown up, frozen and analysed by Southern hybridization of *EcoRI*-digested genomic DNA with flanking probe A, probe B internal to the targeting construct, and probe C specific for the neomycin-resistance gene. **d**, In order to generate germ-line chimaeras, targeted ES clones were injected into BALB/c blastocysts as previously described²⁶ and transferred to pseudopregnant CD1 foster mothers. Chimaeric offspring were identified by eye and coat pigmentation. Offspring with black coat colour originating from the cross between male chimaeras and C57BL/6 females indicated germ-line chimaerism and were tested for the mutation by Southern blot analysis of *EcoRI*-digested tail DNA with flanking probe A. Heterozygous animals were interbred and homozygous perforin 0/0 animals were identified by the same method. **e**, Spleen mRNA from mice infected i.v. 8 days earlier with 200 PFU LCMV-WE was retrotranscribed with oligo(dT) primers and analysed by PCR amplification with the indicated primer pairs. Primer pair a/b amplified two fragments of 472 and 365 bp originating from alternative splicing of perforin 5' untranslated mRNA⁴⁸. Primer pairs c/d, c/f, e/f and the β -actin specific primer pair gave rise to fragments of 481, 898, 350 and 418 bp, respectively. Blank control samples were run for each primer pair to check for false positive signals. **f**, To detect perforin protein, spleen cells from day-8 LCMV-WE-primed mice were restimulated *in vitro* for 5 d with LCMV-infected macrophages. Cell smears of activated bulk cultures were stained with diluted ascites fluid from rat anti-mouse perforin antibody P1-8 (ref. 45). The staining was developed with an alkaline-phosphatase-labelled goat anti-rat immunoglobulin antibody and an alkaline-phosphatase-labelled donkey anti-goat immunoglobulin antibody followed by 5-bromo-4-chloro-3-indolyl phosphate and 4-nitroblue tetrazolium chloride.

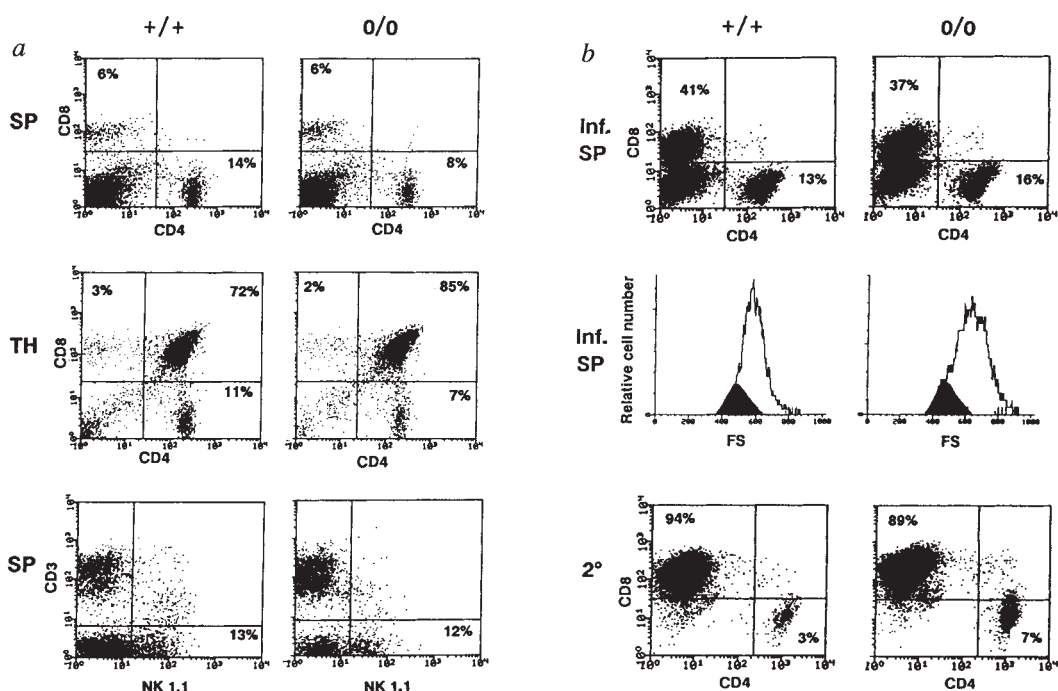
tion is not suppressed from the perforin promoter up to the *neo* cassette in perforin 0/0 mice. If the *neo* cassette had been deleted from the mRNA by splicing, primer pairs c/f and e/f would have amplified the same fragments from normal and perforin 0/0 mice. As perforin 0/0 complementary DNA remained negative, and although both PCR reactions were positive for perforin $+ / +$ control cDNA, splicing of the *neo* cassette does not take place in perforin-deficient mice.

In vitro restimulated LCMV-specific spleen cells from normal mice showed distinct staining of cytoplasmic granula with an anti-perforin antibody, whereas no staining was detected on cells from perforin 0/0 mice (Fig. 1f). Thus the targeted mutation of the perforin gene led to a complete absence of perforin protein.

Inspection of the internal and lymphoid organs of perforin-deficient mice revealed no obvious abnormalities. Based on the observation that granular metrial gland cells in the uterus of

FIG. 2 Lymphocyte populations in lymphoid tissue of normal mice and their change in response to antigenic stimulation. *a*, Normal development of CD8⁺ T cells and NK cells in the absence of perforin. Lymphocytes from spleens (SP) and thymus (TH) were analysed for the expression of T-cell markers CD4, CD8, CD3, and NK marker NK1.1 by cytofluorometry. NK cells were detected by a triple staining with IgM, CD3 and NK1.1-specific antibody. CD3 and NK1.1 staining is shown for cells electronically gated on IgM-cells. Percentages are given for dot-blot quadrants. *b*, Comparable activation and expansion of CD8⁺ T cells in wild-type and perforin-deficient mice upon LCMV infection. Spleen cells were analysed for the expression of CD4 and CD8 8 d after infection with 200 PFU of LCMV-WE (Inf.SP) and after secondary stimulation *in vitro* for 5 d with infected peritoneal macrophages (2°). In addition, the forward scatter of CD8⁺ spleen cells from LCMV-infected mice is compared with the FS of CD8⁺ spleen cells from uninfected animals (black overlay) to show blastformation of activated cells.

METHODS. To analyse T-cell subsets, cells were concomitantly stained



with FITC-conjugated anti-CD8 antibody and PE-conjugated anti-CD4 antibody. Viable cells were gated by a combination of forward and 90° side light-scatter and analysed on a FACSCAN cytofluorometer (Becton Dickinson) using logarithmic scales. NK cells were detected by triple staining with FITC-conjugated anti-IgM Fab fragments, a PE-conjugated anti-CD3 antibody, a biotinylated anti-NK1.1 antibody and a RED-613-streptavidin conjugate.

pregnant mice express perforin, it has been suggested that perforin produced by these cells is somehow involved in the maintenance of pregnancy²⁷. However, perforin-deficient mothers had normal numbers of offspring and did not suffer from embryo-derived tumours, demonstrating that perforin is not necessary for reproduction.

Lymphocytes from spleen and thymus were analysed by cytofluorometry for cell-surface markers CD4, CD8, CD3 and NK1.1 (Fig. 2a). Normal numbers of CD8⁺ cells were found in spleen, and the relative size of the CD8 single-positive, the CD4 single-positive and the CD4/CD8 double-positive populations in the thymus were not significantly different in normal and perforin-deficient mice. Triple staining with anti-IgM, anti-CD3 and anti-NK1.1 antibody showed that the spleen cell population containing NK cells is not affected by the lack of perforin. Also, the frequency of B220⁺ cells in the spleen and blood was within normal ranges (data not shown). These results show that the lack of perforin does not affect differentiation to mature T cells, B cells and NK cells.

Antiviral cytolytic activities *in vitro*

To assess the role of perforin in cytolysis by CTL and NK cells, perforin-deficient and control mice were infected with a low dose (200 PFU) of LCMV-WE, a non-cytopathic virus which is mainly controlled by CD8⁺ CTLs²⁸. Spleen cells from normal mice develop a strong LCMV-specific CTL response that peaks on day 8 after infection (Fig. 3a). In contrast, spleen cells from LCMV-infected perforin-deficient mice did not lyse infected MC57G fibrosarcoma cells (H-2^b), MC57G cells that were labelled with a main epitope peptide of LCMV²⁹, peptide-labelled B16 melanoma cells (H-2^b) and peptide-labelled EL-4 thymoma cells (H-2^b). However, a markedly reduced but specific cytotoxic activity of perforin-deficient cells was detected on peptide-labelled RMA lymphoma cells (H-2^b) (Fig. 3a). The amount of this activity was estimated by graphically comparing

the effector-to-target ratios at equivalent half-maximal ⁵¹Cr-release values to be ~10% of the lysis observed with perforin-expressing effector cells. For MBL-2 (H-2^b), another lymphoma cell line tested in the same way, perforin-independent activity was ~2% of normal lysis (data not shown). To increase antigenic stimulation further, LCMV-primed spleen cells were restimulated *in vitro* with infected macrophages. Cytolytic activities of secondary cultures were high for primed C57BL/6 cells and lacking for perforin-deficient cells on infected and peptide-labelled MC57G cells (Fig. 3b), indicating that the lack of cytotoxicity of perforin-deficient CD8⁺ T cells does not reflect insufficient activation but rather an intrinsic inability to cause specific lysis of target cells.

As immune suppression by high titres of LCMV-WE²⁸ could have complicated the above findings, a second antiviral CTL response was tested. Vaccinia-virus-specific cytotoxicity was absent in perforin-deficient mice after primary infection *in vivo* and after additional secondary stimulation *in vitro* (Fig. 3c), confirming the results obtained with LCMV.

The role of perforin was also tested in NK-cell-mediated cytolysis of sensitive YAC-1 target cells. NK activity was induced by intraperitoneal injection of poly(I-C) or by virus infection. NK-cell-mediated lysis was not evident in the spleen cells of perforin-deficient mice after both types of stimulation (Fig. 3d).

Activation and expansion of CD8⁺ T cells

To exclude the possibility that the absence or reduction of cytotoxicity in perforin-deficient animals was due to a lack of CTL activation, the expansion of CD8⁺ T cells after LCMV infection was followed by cytofluorometry. Normal and deficient animals responded similarly, with a marked increase in CD8⁺ cells from 6 to 37% (compare Fig. 2a and b for 0/0 mice) to LCMV infection and expanded further during secondary restimulation *in vitro* from 37 to 89% (Fig. 2b). In parallel, proliferation of spleen cells primed mainly with CD8⁺ (Fig. 2b) was measured by thymi-

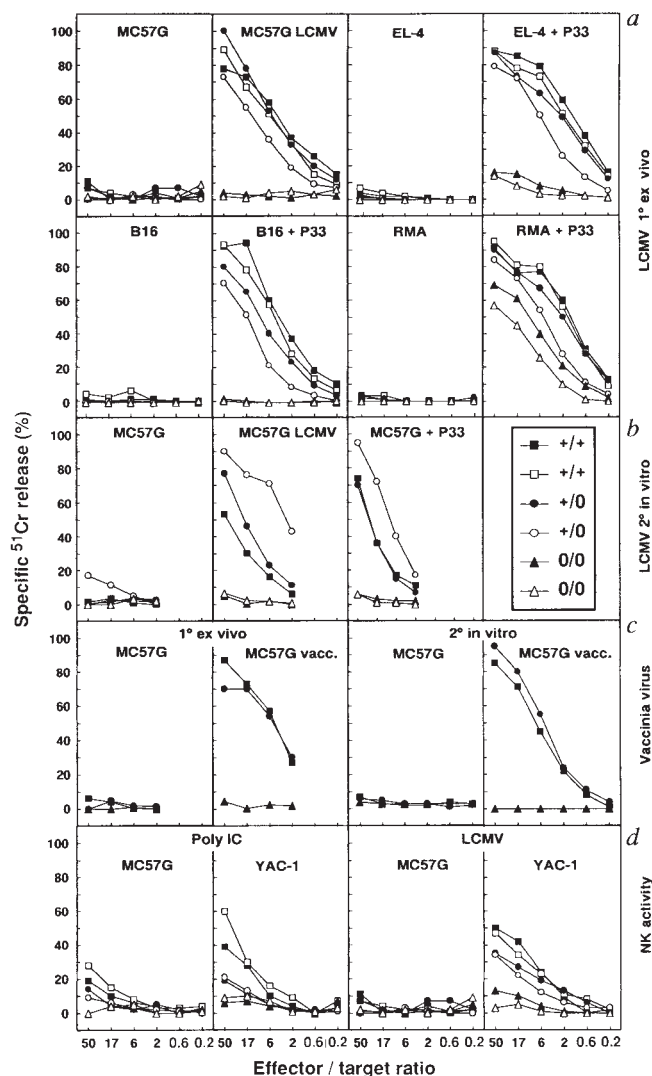


FIG. 3 Anti-LCMV, anti-vaccinia virus and NK cell cytotoxic activity. Anti-viral cytolytic activity of perforin-deficient spleen cells on fibroblast target cells is completely absent not only after primary infection *in vivo* but also after secondary stimulation *in vitro*. Markedly reduced perforin independent activity was observed on target cells of lymphohaematopoietic origin. Cytolytic NK activity is completely absent from perforin-deficient animals as well. Individual normal (■), heterozygous (●) and perforin-deficient (▲) mice are denoted by open and filled symbols. The lytic activities of primary and secondary effector cells were determined in ^{51}Cr -release assays with 5 h incubation time as described¹. **a**, Cytotoxicity 8 d after primary acute infection of mice with LCMV-WE (200 PFU i.v.). $\text{H}-2^b$ syngeneic target cells were MC57G fibroblasts, B16 melanoma cells and RMA lymphoma cells. Target cells were either uninfected, infected with LCMV-WE or labelled with peptide P33 of LCMV-GP (amino acids 33–41 KAVYNFATM of LCMV-GP), which is a main epitope of LCMV in the $\text{H}-2^b$ haplotype²⁹. **b**, Cytotoxicity after additional LCMV specific secondary stimulation *in vitro*. Spleen lymphocytes from LCMV primed mice were restimulated *in vitro* for 5 d with LCMV-WE-infected peritoneal macrophages before being assayed as in **a**. **c**, Anti-vaccinia virus cytotoxic T-cell activity was assessed with spleen lymphocytes from mice infected with 2×10^6 PFU vaccinia virus Lancy (Serum und Impfstoffinstitut, Bern) 6 d previously either directly *ex vivo* or after additional culture for 5 d with vaccinia virus-infected macrophages. Target cells were uninfected MC57G ($\text{H}-2^b$) fibroblasts and fibroblasts infected with vaccinia virus Lancy. **d**, Natural killer cell activity in perforin-deficient mice. Natural killer cells were induced by intravenous injection of $100 \mu\text{g}$ poly(I:C) 24 h before spleen cells were tested on NK-sensitive YAC-1 and non-sensitive MC57G fibroblast target cells. In addition, the NK activity of spleen cells still measurable 8 d after infection with LCMV-WE (200 PFU, i.v.) was determined (LCMV).

dine incorporation upon secondary restimulation with LCMV-infected macrophages. The stimulation indices of 8.5 for perforin-deficient animals and 7.25 for normal mice (data not shown) were not significantly different. Also, forward light scatter (FS) of CD8^+ spleen cells during LCMV infection indicated that blast formation and activation was comparable in both perforin-deficient and normal mice (Fig. 2b). Thus, activation and proliferation of specific CD8^+ T cells was not affected by the absence of perforin.

Alloreactivity

The role of perforin in T-cell-mediated alloantigen-specific cytotoxicity, particularly in peritoneal exudate lymphocytes (PEL), has been questioned³⁰. Therefore, we tested major histocompatibility complex (MHC) alloantigen-specific cytotoxicity of C57BL/6 anti-BALB/c mixed lymphocyte cultures, primary *in vivo*-stimulated spleen and PEL effector cells. CTLs from normal and heterozygous animals generated in primary mixed lymphocyte cultures expressed high cytolytic activity on all tested $\text{H}-2^d$ target cell lines (D2 fibroblasts, P815 mastocytoma and L1210 T cell lymphoma), but not on syngeneic EL-4 lymphoma target cells (Fig. 4a). In contrast, perforin-deficient cultures failed to lyse D2 fibroblasts, and lysis of P815 mastocytoma and L1210 lymphoma target cells was reduced to ~10% of that of perforin-expressing CTLs. To test the cytotoxicity of primary effector cells generated *in vivo*, mice were immunized with allogeneic P815 mastocytoma cells intraperitoneally. After 13 days, spleen cells and PELs were collected. The numbers of peritoneal cells recovered from perforin-expressing and perforin-deficient animals did not vary significantly, so the perforin-deficient mice were able to control growth of the injected P815 cells by day 13 after inoculation (data not shown). Normal mice efficiently lysed allogeneic D2, BALB/c mouse embryonal fibroblasts (MEF) and P815 cells, whereas spleen cells and PELs from perforin-deficient animals showed no cytolytic activity on D2 fibroblasts and BALB/c MEFs. Lysis of P815 cells (Fig. 4b, c) was reduced to an extent comparable to that shown in Fig. 4a. Interestingly, lysis by heterozygous cells of D2 and BALB/c MEF was less effective than in normal C57BL/6 animals, indicating a perforin gene dosage effect on lysis of fibroblast target cells (Fig. 4b, c).

Cytotoxic effector functions *in vivo*

Clearance of LCMV infection and the immunopathological phenomena induced by this virus are essentially mediated by CD8^+ T cells^{2,28}, providing a model system for evaluating the functions of perforin *in vivo*. Normal and perforin-deficient mice were infected intravenously (i.v.) with a low dose of LCMV-WE and virus titres were determined in various organs 12 days after infection. Normal and heterozygous mice had completely eliminated the virus by that time in all organs (Table 1). In contrast, perforin-deficient mice still had high titres of virus in spleen and liver and lower titres in kidney and brain. Eventually, infection i.v. or in the foot led to weight loss and death between days 13 and 16 (Fig. 5a). The cause for this condition, which resembles chronic wasting, is unclear, but could be explained by a continued confrontation of specific T cells with virus-infected cells resulting in massive secretion of cachectic factors.

A physiological *in vivo* correlate of the ^{51}Cr -release assay has been described³¹. In this system, the increase of liver-specific transaminases and glutamate dehydrogenase in the serum directly reflects the action of cytolytic T cells during LCMV-induced aggressive hepatitis. Upon injection of 5×10^4 PFU LCMV-WE, normal mice developed hepatitis with LCMV-infected hepatocytes, CD8^+ T-cell infiltrates, liver cell necrosis and elevated amounts of glutamate dehydrogenase in the serum. In contrast, histological examination of livers from perforin-deficient animals revealed LCMV-infected hepatocytes and CD8^+ T-cell infiltrates comparable to levels in normal control mice (Fig. 5f), but no significant increase in glutamate dehydrogenase (Fig. 5d). Upon low-dose (200 PFU) i.v. infection with

TABLE 1 Virus titres of C57BL/6, heterozygous and perforin-deficient mice 12 days after infection with a low dose of LCMV-WE

Genotype	Spleen (PFU g ⁻¹)	Liver (PFU g ⁻¹)	Kidney (PFU g ⁻¹)	* Brain (PFU g ⁻¹)	Blood (PFU ml ⁻¹)
+/+	<200	<200	<200	<200	<200
+/+	<200	<200	<200	<200	<200
+ / 0	<200	<200	<200	<200	<200
+ / 0	<200	<200	<200	<200	<200
0/0	3.8×10^7	1.0×10^6	1.1×10^3	9.0×10^2	3.0×10^4
0/0	2.5×10^7	1.0×10^6	4.2×10^5	2.5×10^3	6.5×10^5

Mice were infected with 200 PFU of LCMV isolate WE and killed on day 12. Viral titres of the various organs of individual mice were determined by a focus-forming assay⁴⁴ and are given as plaque-forming units per gram of tissue from one of two similar experiments.

LCMV-WE, perforin 0/0 mice developed CD8⁺ T-cell infiltrates and virus-infected hepatocytes as in high-dose infection; normal controls showed only a small amount of CD8⁺ T-cell infiltration (data not shown) and few virus-infected cells in the liver, confirming the results shown in Table 1. No lymphocyte infiltrates

were observed in livers of uninfected perforin 0/0 and control mice (data not shown).

Upon LCMV injection into the footpad of normal mice, a local inflammatory swelling reaction develops that is mediated sequentially by CD8⁺ and CD4⁺ T cells³². In perforin-deficient mice, footpad swelling failed to develop up to day 16, whereas control mice responded promptly (Fig. 5b).

After intracerebral inoculation of LCMV, normal mice die from a lethal choriomeningitis mediated mainly by CD8⁺ T cells^{2,28}. CD4⁺ T cells may cause disease and weight loss that is only rarely lethal^{33,34}. Perforin-deficient mice survived intracerebral infection with 200 PFU LCMV-WE (Fig. 5c), but suffered from a weight loss of 15–20%. Occasionally, infection with the more neurotropic strain LCMV-ARM caused delayed death of some perforin-deficient mice without inducing the characteristic spastic paresis. Although these preliminary results require further investigation, they suggest that in LCMV-triggered T-cell-dependent choriomeningitis, lytic components may cause the early aggressive symptoms of the disease whereas non-lytic pathogenetic components may be responsible for the later, more chronic course of the disease observed in the absence of cytotoxic T cells.

Immune surveillance against syngeneic MC57G fibrosarcoma tumour cells predominantly depends on CD8⁺ T cells, but CD4⁺ T cells and NK cells are also involved³⁵. Upon inoculation of 10⁶ tumour cells into the foot of normal mice, the tumour cells grew to some extent up to day 12, then they were rejected. Perforin 0/0 mice were considerably more susceptible to the effects of MC57G cells; all of the perforin-deficient mice inoculated with a dose of 10⁶ cells failed to reject (Fig. 5e), whereas normal control mice developed tumours only when more than 3 × 10⁶ MC57G cells were injected. One out of two perforin-deficient mice even showed tumour growth when as few as 10⁵ MC57G cells were inoculated (data not shown).

Discussion

Specifically activated CD8⁺ T cells from perforin-deficient mice, including the debated PELs, cannot lyse *in vitro* virus-infected, peptide-coated or allogeneic target cells of epithelial, neuroectodermal or mesodermal origin. A minor lytic activity is detected, however, on some lymphohaematopoietic target cells. Also, the cytolytic activity of NK cells depends on perforin. These results formally show that perforin is an essential part of the main mechanism of cell lysis by cytotoxic lymphocytes, but cannot prove whether perforin alone is sufficient to induce lysis in target cells. As purified perforin can lyse target cells, perforin alone may in fact be sufficient.

Our results show that a perforin-mediated mechanism accounts for 100% of the observed lysis on most lymphohaematopoietic target cells and for ~90% on some. The specific lytic activity of perforin-deficient effector cells (Figs 3a and 4a–c) is probably due to one or several less effective alternative lytic mechanisms. Although full-length perforin, conceivably originating from splicing out of the *neo* cassette, is not produced in 0/0 mice, we cannot exclude the possibility that a partial, amino-terminal fragment translated from the disrupted perforin gene could express some lytic activity and so account for lysis of lymphohaematopoietic target cells. But we consider this unlikely

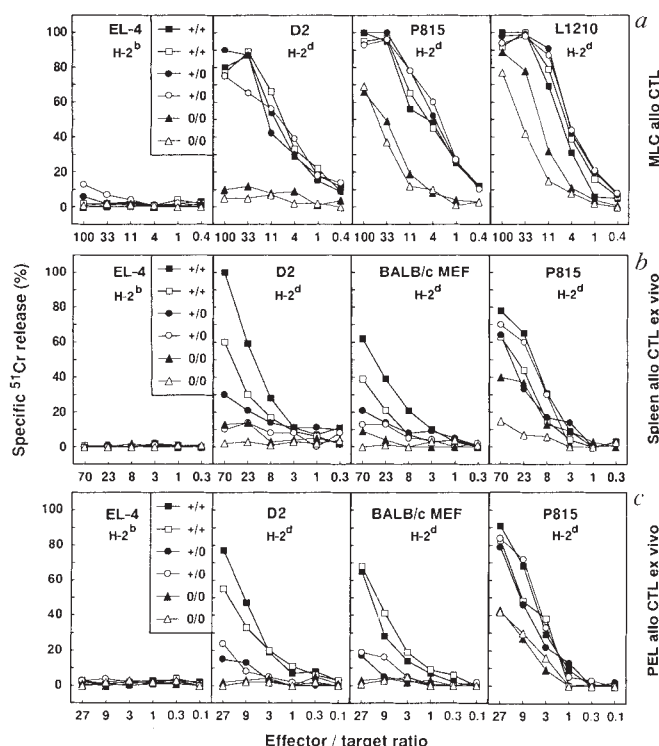
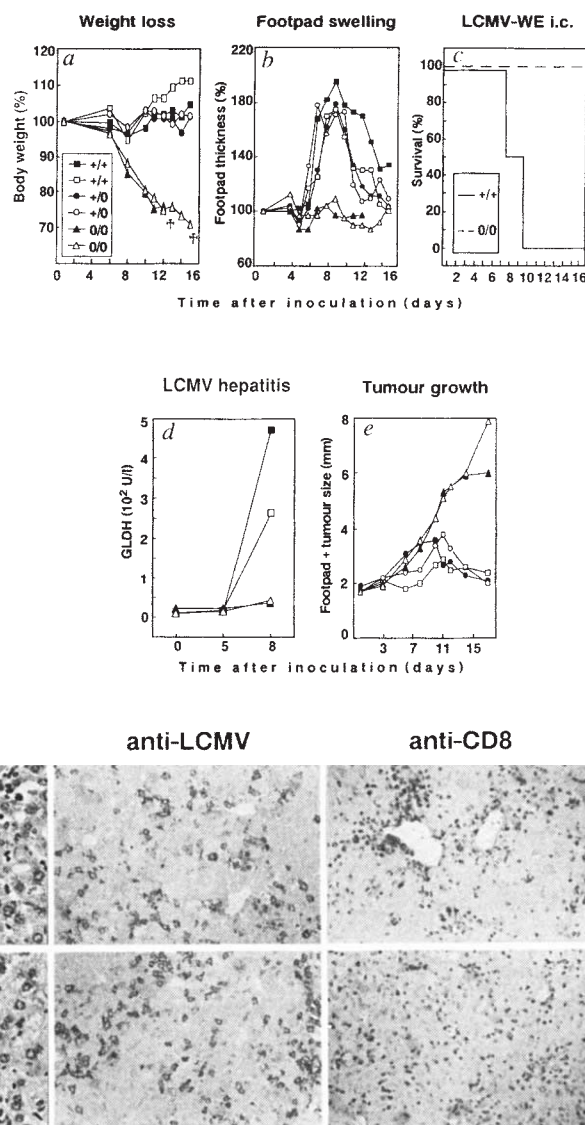


FIG. 4 MHC alloantigen-specific T-cell activity generated in mixed lymphocyte cultures or *in vivo*. Individual normal control (■), heterozygous (●) and perforin-deficient (▲) mice are denoted by open and filled symbols. a, Alloantigen-specific cytotoxicity generated in mixed lymphocyte cultures *in vitro*. Spleen cells from unprimed mice were stimulated in MLCs with irradiated BALB/c stimulator spleen cells. After 5 d, the CTL activity was tested by ⁵¹Cr-release of EL-4 (lymphoma, H-2^b), D2 (fibrosarcoma, H-2^d), BALB/c MEF (BALB/c untransformed embryonal fibroblasts, H-2^d), P815 (mastocytoma, H-2^d) and L1210 (lymphoma, H-2^d) target cells. b, H-2^d alloantigen-specific cytotoxic activity of spleen cells activated *in vivo* by i.p. injection of 3 × 10⁷ allogeneic P815 cells. After 13 d, spleens and PELs (c) were used for evaluation of cytotoxicity on the indicated target cells. c, Alloantigen-specific cytotoxic activity of peritoneal exudate lymphocytes obtained by peritoneal lavage from the same mice analysed in b.

METHODS. a, Embryonal fibroblasts were obtained by culturing single-cell suspensions from trypsinized day-14 to day-19 BALB/c embryos. b, Primary allospecific peritoneal and spleen effector cells were prepared from the same mice 13 d after inoculation with 3 × 10⁷ P815 cells into the peritoneum. At this time point, cytofluorometric analysis for MHC class I antigen expression showed that allogeneic cells were eliminated from the peritoneum of normal and perforin-deficient animals, thus eliminating the possibility of cold target inhibition by the presence of unlabelled target cells during the ⁵¹Cr-release assay. P815 cells were grown by weekly peritoneal passage in female DBA/2 mice.

FIG. 5 Consequences of the lack of cytotoxic activity *in vivo*. Individual normal control (■), heterozygous (●) and perforin-deficient (▲) mice are denoted by open and filled symbols. **a**, Weight loss of perforin-homozygous mutant animals and death between day 13 and day 16 upon infection with 200 PFU of LCMV-WE into the foot. **b**, Absence of LCMV-induced primary swelling reaction in mice infected with 200 PFU of LCMV-WE in the foot. Footpad thickness was measured daily with a spring-loaded caliper. Swelling was calculated as per cent of footpad thickness on day 1 after infection. Values are given as means of both footpads of one mouse. **c**, Choriomeningitis induced by intracerebral injection of 200 PFU LCMV-WE. After injection, groups of four mice were monitored daily for lethality, clinical signs of choriomeningitis and reduction of body weight. **d**, Detection of cytolytic activity *in vivo* by the increase of liver-specific enzyme GLDH indicating hepatic immunopathology upon i.v. infection with 5×10^4 PFU LCMV-WE. Mononuclear liver infiltrates were similar in perforin 0/0 and normal control mice (**f**). Glutamate dehydrogenase (GLDH; EC1.4.3.) was determined by a standard method as described previously³¹. **e**, Resistance to injected fibrosarcoma tumour cells. Values given are thicknesses of single footpads from different mice upon injection of 10^6 MC57G fibrosarcoma tumour cells into the footpad. In accord with regulations, mice were killed when footpad thickness reached more than 6–8 mm. **f**, Infiltration of mononuclear cells into the liver 8 d after i.v. infection with 5×10^4 LCMV-WE.

METHODS. **f**, Liver sections were stained with haematoxylin/eosin, rabbit anti-LCMV serum or with rat monoclonal anti-CD8 antibody 169 (ref. 49). Immunohistology was developed with alkaline phosphatase-labelled goat anti-rabbit or goat anti-rat antibodies followed by alkaline phosphatase-labelled donkey anti-goat antibodies, A-BI phosphate and New Fuchsin.



because (1) if a lytic fragment of perforin was effective in perforin 0/0 effector cells, then those target cells that are most sensitive to perforin-independent lysis would be expected to be most sensitive to lysis by isolated granules and perforin protein. This is clearly not the case, because P815 mastocytoma cells, which are sensitive to lysis by perforin 0/0 effector cells, are much less sensitive to lysis by isolated granules than the perforin 0/0 resistant EL4 thymoma target cells³⁶; and (2) target cells sensitive to lysis by perforin 0/0 effectors would be expected to be lysed more efficiently by normal effector cells. As can be seen in Figs 3a and 4, this is not the case.

Therefore, specific lysis of some target cells by perforin-deficient T cells suggests that less effective alternative lytic pathways exist which act mainly on certain lymphohaematopoietic cells. As observed earlier, the features of lysis vary among different types of target cells: for example, morphological signs of apoptosis have been described in untransformed macrophages but not in transformed fibroblasts³⁷, and fragmentation of chromatin into oligonucleosomes indicating programmed cell death occurs during lysis of lymphoid cells but not of fibroblasts^{38,39}. The availability of perforin-deficient CTLs will enable these alternative cytolytic mechanisms to be investigated. Free Ca^{2+} -independent cytotoxicity may be mediated by the surface molecule Fas⁶, but further investigation is needed to clarify whether Fas can also account for perforin-independent target cell lysis.

It was observed that allogeneic P815 mastocytoma cells in the

peritoneum were eliminated in the absence of perforin but that elimination of syngeneic, subcutaneously growing MC57G fibrosarcoma cells was dependent on perforin. This could be the result of either perforin-independent lytic T-cell mechanisms or of a general inflammatory process with participation of macrophages and granulocytes.

Our second key finding is that clearance of LCMV by CD8^+ T cells depends on perforin-mediated cytolytic activity. Thus other contact-dependent signals or secretion of soluble factors must be less important in anti-LCMV immune defence, although this finding is not generalizable to all viruses: for example, vaccinia virus may be controlled by interferons released by T cells^{4,40} as well as by CD8^+ T-cell-mediated cytotoxicity⁴¹.

Surprisingly, perforin 0/0 mice failed to develop any footpad swelling reaction after LCMV infection in the foot. This is in contrast to results with CD8 -deficient mice³³, but parallels those from β_2 -microglobulin-deficient mice⁴². One explanation could be that without lysis of infected cells, not enough soluble viral antigen is released for presentation to CD4^+ T cells to trigger a local inflammatory process. Alternatively, the rapid spread of LCMV to tissues outside the foot could preclude the formation of an antigen gradient and 'dilute' the effector T cells, so preventing migration of T cells to the foot and hence footpad swelling.

Cytotoxic T cells are involved in the recognition of tumour antigens and tumour rejection^{1,43}. We have shown that perforin-dependent cytolytic activity is an important component of this

effector function, as perforin-deficient mice have a considerably reduced capacity to eliminate MHC class-I-positive, class-II-negative MC57G fibrosarcoma tumour cells.

Because mice without perforin are unable to generate cytolytic CTLs and NK cells, these animals should provide a useful model

for assessing the contribution of cytolytic activity in microbial infections, tumour resistance and autoimmune diseases. Particularly, they should allow differentiation between effector functions mediated by secreted factors and those mediated by the cytolytic activity of CD8⁺ T cells and NK Cells. □

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1. Cerottini, J. C. & Brunner, K. T. *Adv. Immun.* **18**, 67–132 (1974).
2. Lehmann-Grube, F., Moskopidis, D. & Löhler, J. *Ann. N. Y. Acad. Sci.* **532**, 238–256 (1988).
3. Martz, E. & Howell, D. M. *Immun. Today* **10**, 79–86 (1989).
4. Ramshaw, I., Ruby, J., Ramsay, A., Ada, G. & Karupiah, G. *Immunol. Rev.* **127**, 157–182 (1992).
5. Russell, J. H. *Immunol. Rev.* **72**, 97–118 (1983).
6. Rouvier, E., Luciani, M. F. & Golstein, P. *J. exp. Med.* **177**, 195–200 (1993).
7. Jenne, D. E. & Tschopp, J. *Immunol. Rev.* **103**, 53–71 (1988).
8. Dourmashkin, R. R., Deteix, P., Simone, C. B. & Henkart, P. *Clin. exp. Immun.* **42**, 554 (1980).
9. Dennert, G. & Podack, E. R. *J. exp. Med.* **157**, 1483–1495 (1983).
10. Young, J. D. E., Hengartner, H., Podack, E. R. & Cohn, Z. A. *Cell* **44**, 849–859 (1986).
11. Henkart, P. A. *Rev. Immun.* **3**, 31–58 (1985).
12. Podack, E. R., Ding-E Young, J. & Cohn, Z. A. *Proc. natn. Acad. Sci. U.S.A.* **82**, 8629–8633 (1985).
13. Acha-Orbea, H., Scarpellino, L., Hertig, S., Dupuis, M. & Tschopp, J. *EMBO J.* **9**, 3815–3819 (1990).
14. Shiver, J. W., Su, L. & Henkart, P. A. *Cell* **71**, 315–321 (1992).
15. Lichtenheld, M. et al. *Nature* **335**, 448–451 (1988).
16. Shinkai, Y., Takio, K. & Okumura, K. *Nature* **334**, 525–527 (1988).
17. Podack, E. R. et al. *Immun. Rev.* **103**, 203–211 (1988).
18. Garcia-Sanz, J. A. et al. *EMBO J.* **6**, 933–938 (1987).
19. Kawasaki, A., Shinkai, Y., Yagita, H. & Okumura, K. *Eur. J. Immun.* **22**, 1215–1219 (1992).
20. Mueller, C. et al. *Eur. J. Immun.* **19**, 1253–1259 (1989).
21. Mueller, C. et al. *J. exp. Med.* **167**, 1124–1136 (1988).
22. Young, L. H. et al. *Lancet* **336**, 1019–1021 (1990).
23. Trapani, J. A. et al. *J. exp. Med.* **171**, 545–557 (1989).
24. Podack, E. R., Hengartner, H. & Lichtenheld, M. G. A. *Rev. Immun.* **9**, 129–157 (1991).
25. Thomas, K. R. & Capecchi, M. R. *Cell* **51**, 503–512 (1987).
26. Ledermann, B. & Bürki, K. *Exp. Cell Res.* **197**, 254–258 (1991).
27. Parr, E. L., Young, L. H. Y., Parr, M. B. & Young, J. D. E. *J. Immun.* **145**, 2365–2372 (1990).
28. Buchmeier, M. J., Welsh, R. M., Dutko, F. J. & Oldstone, M. B. A. *Adv. Immun.* **30**, 275–331 (1980).

29. Pircher, H. P. et al. *Nature* **346**, 629–633 (1990).
30. Berke, G. & Rosen, D. *Transplant. Proc.* **19**, 412–416 (1987).
31. Zinkernagel, R. M. et al. *J. exp. Med.* **164**, 1075–1092 (1986).
32. Moskopidis, D. & Lehmann-Grube, F. *Proc. natn. Acad. Sci. U.S.A.* **86**, 3291–3295 (1989).
33. Fung-Leung, W. P., Kündig, Th. M., Zinkernagel, R. M. & Mak, T. W. J. *J. exp. Med.* **174**, 1425–1429 (1991).
34. Muller, D. et al. *Science* **255**, 1576–1578 (1992).
35. Kohler, M., Rüttner, B., Cooper, S., Hengartner, H. & Zinkernagel, R. M. *Canc. Immun. Immunother.* **32**, 117–124 (1990).
36. Podack, E. R. *Immun. Today* **6**, 21–27 (1985).
37. Baenziger, J., Hengartner, H., Zinkernagel, R. M. & Groscurth, P. *Scand. J. Immun.* **28**, 411–423 (1988).
38. Howell, D. M. & Martz, E. J. *Immun.* **138**, 3695–3698 (1987).
39. Sellins, K. S. & Cohen, J. J. *J. Immun.* **147**, 795–803 (1991).
40. Huang, S. et al. *Science* **259**, 1742–1745 (1993).
41. Binder, D. & Kündig, Th. M. *J. Immun.* **146**, 4301–4307 (1991).
42. Lehmann-Grube, F., Löhler, J., Utermöhlen, O. & Gegin, C. J. *Virology* **67**, 332–339 (1993).
43. Melief, C. J. M. *Adv. Cancer Res.* **58**, 143–175 (1992).
44. Battegay, M. et al. *J. Virol. Meth.* **33**, 191–198 (1991).
45. Kawasaki, A. et al. *Int. Immun.* **2**, 678–684 (1990).
46. Mansour, S. L., Thomas, K. R. & Capecchi, M. R. *Nature* **336**, 348–352 (1988).
47. Stewart, C. L., Schuetz, S., Vanek, M. & Wagner, E. F. *EMBO J.* **6**, 383–388 (1987).
48. Yoon, B. S. et al. *J. exp. Med.* **173**, 813–822 (1991).
49. Cobbold, S. P., Jayasuriya, A., Nash, A., Prospero, T. D. & Waldmann, H. *Nature* **312**, 548–551 (1984).

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LETTERS TO NATURE

Fullerenes in an impact crater on the LDEF spacecraft

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THE fullerenes C₆₀ (ref. 1) and C₇₀ have been found to occur naturally on Earth^{2,3}, and have also been invoked to explain features in the absorption spectra of interstellar clouds^{1,4}. But no definitive spectroscopic evidence exists for fullerenes in space and attempts to find fullerenes in carbonaceous chondrites have been unsuccessful^{5,6}. Here we report the observation of fullerenes associated with carbonaceous impact residue in a crater on the Long Duration Exposure Facility (LDEF) spacecraft. Laser ionization mass spectrometry and Raman spectroscopy indicate the presence of fullerenes in the crater and in adjacent ejecta. Man-made fullerenes survive experimental hypervelocity (~6.1 km s⁻¹) impacts into aluminium targets, suggesting that space fullerenes contained in a carbonaceous micrometeorite could have survived the LDEF impact at velocities towards the lower end of the natural particle encounter range (<13 km s⁻¹). We also demonstrate that the fullerenes were unlikely to have formed as instrumental artefacts, nor are they present as contaminants. Although we cannot specify the origin of the fullerenes with certainty, the most plausible source is the chondritic impactor. If, alternatively, the impact produced the fullerenes *in situ* on LDEF, then this suggests a viable mechanism for fullerene production in space.

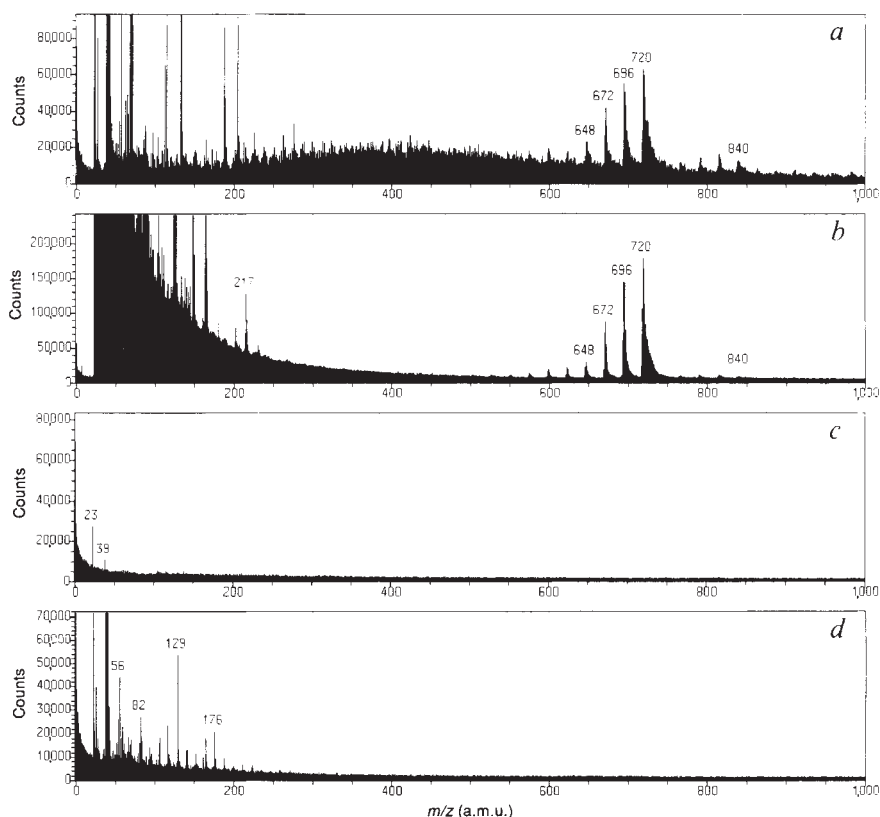
We used laser ionization time-of-flight mass spectrometry (LIMS) to study the carbon-rich regions in and around a crater (no. 31) in an aluminium panel on the LDEF spacecraft. The LIMS spectrum of an area of the raised crater rim shows major peaks at mass-to-charge ratios (*m/z*) of 720 and 840, C₆₀⁺ and C₇₀⁺, respectively (Fig. 1a). Peaks separated by successive 24-dalton increments (C₂) are typical of fullerenes. We obtained virtually identical results from samples of commercially available fullerenes analysed under similar instrumental conditions (Fig. 1b). The mass spectrum acquired from the blank aluminium surface ~0.2 mm away from the crater (Fig. 1c) is typical of that taken at many spots and demonstrates that fullerenes are localized in and around the crater.

Figure 2 shows a scanning electron micrograph of this crater (no. 31), which was found in the aluminium panels of the passive collector tray (chemistry of micrometeoroid experiment) located in row 11 near the vehicle's leading edge⁷. The impact crater was probably formed by a carbonaceous impactor^{8,9}. Auger electron spectroscopy and scanning Auger microscopy surface analyses of the crater bottom and an area immediately adjacent to the crater (~0.02 mm from crater rim) showed carbon concentrations of 40% and ~13%, respectively⁸. Micro-Raman spectroscopy confirms that the carbon exists mainly in a combination of amorphous and graphitic states, but observable signals from fullerenes are also present (Fig. 3). Carbon was found in the crater around the edges and in the vicinity outside the crater. Energy dispersive X-ray spectrometry of the residue on the crater bottom^{8,10} showed a roughly chondritic composition of other elements (Si, Ca, Mg, Fe) indicating a probable extraterrestrial origin for the impactor. The presence of these elements, over and above the composition of the aluminium plate, is confirmed here (see Table 1).

We considered several scenarios that could produce fullerene signals as artefacts. We first examined instrumental memory

FIG. 1 *a–d*, Mass spectra obtained by the laser ionization time-of-flight mass spectrometry (LIMS) technique. *a*, Spectrum from the rim of the LDEF crater. Major peaks occur at mass-to-charge (m/z) ratios of 720 (C_{60}^+) and 840 (C_{70}^+). *b*, Spectrum of synthetic fullerene (Aldrich, catalogue number 37,712-0). The original material has a C_{60}/C_{70} ratio of 9:1. Purification by toluene dissolution and recrystallization resulted in the formation of almost pure C_{60} crystals. The diagnostic peak of C_{60} at m/z 720 can be observed, along with fragmentation peaks at m/z 648, 672, 696 and a weak C_{70} peak at m/z 840. *c*, Spectrum taken at a point remote from the rim of the LDEF crater. No fullerene peaks are observed. *d*, Spectrum acquired from a graphite planchette, same conditions as *a*. No fullerene peaks are observed. Molecular fragmentation of fullerenes is approximately the same for the crater region and for the synthetic material. Laser power density cannot be controlled to the point of eliminating molecular fragmentation. Also, small differences in optical properties of the target material can significantly affect the absorption of laser light, and hence the fragmentation. The low-mass region of both fullerene spectra (*a*, *b*) exhibits substantial signals, mostly inorganic (Na, Al, and K). The spectra of perylene (not shown) acquired under essentially the same instrumental conditions did not produce appreciable molecular fragmentation and little, if any, inorganic contamination was noted. The difference between the fullerene and perylene spectra may be due to the greater complexity of the carbonaceous crater ejecta relative to the simplicity of the perylene molecule.

METHODS. The LIMS¹⁵ technique used a pulsed (~ 5 ns) laser (266 nm, fourth harmonic Nd:YAG) operated at low laser power density ($\sim 5 \times 10^6$ W cm⁻²). Most aromatic species absorb 266-nm radiation. Absorption of a second photon usually provides sufficient energy to ionize the aromatic molecules. If no more photons are absorbed, there is little excess energy for fragmentation of the molecular ion. This variant of resonance enhanced multiphoton ionization REMPI¹⁶ depends on minimal irradiance to prevent absorption of excess energy. The low-irradiance conditions produce little fragmentation of organic molecules,



but also weak ion signals. Therefore, the data reported here are sums of spectra accumulated from 150 laser pulses. We estimate the sensitivity of the LIMS system to fullerenes by analogy with results of fly-ash analysis for polycyclic aromatic hydrocarbons (PAHs). Single particles of fly-ash (~ 0.01 mm diameter) coated with a perylene (molecular weight 252) solution to a bulk value of ~ 140 μ g per g of flyash ($\sim 6 \times 10^{-7}$ mol of perylene per g) were analysed in the LIMS instrument using conditions essentially identical to those used in the fullerene experiments. Relatively intense signals (20 times background noise) of perylene were observed on a single particle. For a calculated particle volume of 5×10^{-13} cm³ and an assumed particle density of 1.5 g cm⁻³, the amounts of perylene detected on single particles were of the order 4×10^{-16} mol. Both PAHs and fullerenes have strong ultraviolet absorption bands near 266 nm (ref. 4), hence it is reasonable to assume that fullerenes have REMPI ion yields comparable to those of PAHs.

effects. Although our LIMS instrument had been used in the analysis of one fullerene sample a few months before the acquisition of the present data, several analyses carried out under the same instrumental conditions before the LDEF data did not reveal any signals around m/z 720 or 840, confirming that memory effects from previous samples are not present. We ruled out sample cross-contamination based on the sample handling history (see Fig. 2), on the basis that a companion crater (no. 74) was found not to contain fullerenes, and because the fullerene signature comes only from the crater rim and from the dark-coloured rays (ejecta) extending radially outward from the crater. The fullerene signature does not come from the surrounding bare aluminium. Thus the fullerenes are closely associated with the crater.

A third scenario is that the signals at m/z 720 and 840 are artefacts of the laser ionization technique. High laser irradiances can induce carbon cluster ion (C_n^+) patterns with n extending from 1 to over 100, including small amounts of C_{60}^+ . The C_{60}^+ intensity can be high when ion beam expansion conditions are optimized for ion clustering and thermalization¹¹. But the LIMS instrument does not operate in this regime, and does not produce C_{60}^+ from organic materials (polymers such as polyethylene

terephthalate (PET), polymethyl methacrylate (PMMA) and nylon) or various types of carbon in the low-power mode described above. LIMS data from pure graphite acquired under the low-irradiance conditions used in this analysis demonstrate that fullerenes are not formed. The relevant spectrum is shown in Fig. 1*d*.

Micro-Raman spectroscopy indicates that the mass spectroscopic results are not due to laser irradiation artefacts. Although the signals are weak, the Raman spectra (Fig. 3) indicate that up to 5% of the carbon associated with the crater is in the form of fullerenes. Raman spectra were measured at several locations in and around crater no. 31. Most of the measurements are of irregularly shaped, raised black areas 0.01–0.04 mm in diameter on the bottom of the crater, or dark regions on the crater rim. Some measurements of the aluminium surface were also obtained as background. Strong fluorescence from some spots precluded useful Raman measurements. Spectra of the dark regions generally included bands at 1,360 and 1,580 cm⁻¹ that are attributed to disordered or poorly graphitized carbon. In some spectra one also observes weak bands at 1,469 and 1,568 cm⁻¹ which are wavenumbers at which common fullerenes absorb¹². These weak signals become more apparent when the

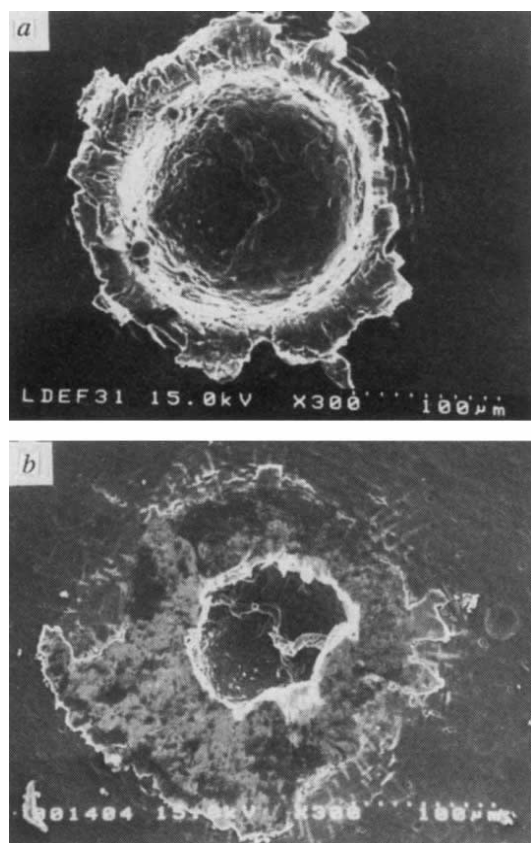


FIG. 2 *a*, Scanning electron micrograph of LDEF crater (no. 31). *b*, Same crater after flattening of raised portions (see Table 1). Ropey material in crater bottoms of both images is impact-fused target and impactor. Dark portions are areas of carbonaceous enrichment. The fullerene-bearing crater no. 31 and crater no. 74 (no detectable fullerenes) came from an aluminium panel which was part of the LDEF experiment tray (F. Hörz, personal communication). After the retrieval of LDEF from space, inspection of the intact vehicle took place in the Kennedy Space Center SAEF II clean room facility. From the time of initial inspection to the time the LDEF was dismantled and the trays were shipped to the respective investigators, the clean room was maintained at the class 100,000 level¹⁷. Both craters were removed from the A1 panel by 'dry coring' 7 mm diameter disks which contained one crater in each disk. This operation was performed by D. Brownlee at the University of Washington under clean room class 100 conditions. The two disks were kept together in sealed glass containers at all times during storage. Both craters were exposed to very similar conditions during instrumental analyses. (Dotted scale bar, 100 µm in *a* and *b*).

carbon spectrum is subtracted. Two spectra with evidence of fullerenes from the rim of crater no. 31 are shown in Fig. 3*a, b*. The same carbon background is subtracted from both spectra. The bottom spectrum (*c*) is that of a reference fullerene sample. The characteristic peaks at 1,430, 1,448, 1,469 and 1,568 cm⁻¹ confirm the presence of fullerenes in crater no. 31.

Two relevant questions need to be addressed. (1) Can fullerenes be formed by high-speed impact of carbon on aluminium? (2) Can pre-existing fullerenes survive high-speed impact? To try to answer these questions, we conducted a series of hypervelocity impact experiment using the NASA Ames two-stage light-gas gun⁹. Impact craters from particles of diamond, graphite, amorphous carbon, Murchison meteorite matrix and an organic crystal (phthalic acid) were examined. The particles (0.05–0.2 mm diameter) were launched towards an aluminium plate under vacuum at speeds from 5.4 to 6.3 km s⁻¹. LIMS spectra indicate that no fullerenes were formed by these impacts, within LIMS detection limits. The fragment ion signature of phthalic acid is observed as well as most PAH signatures in the

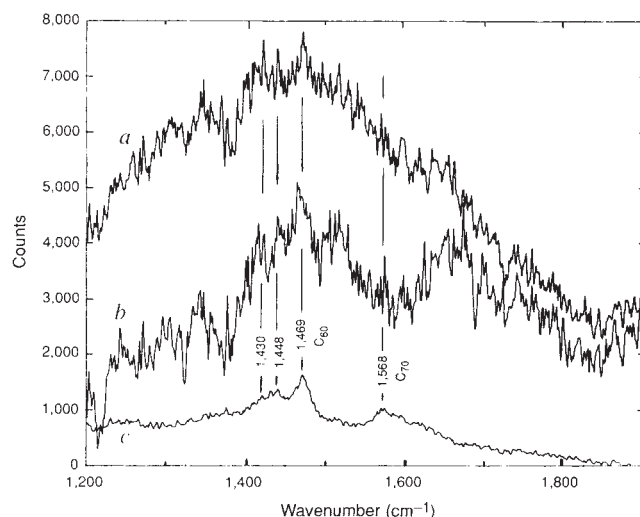


FIG. 3 Micro-Raman spectra from locations on the rim of crater no. 31 (*a, b*), and from reference fullerene (*c*). The Raman spectra were measured using the 488.0 nm line of an argon ion laser (Innova 90, Coherent Radiation Inc.). The laser power was between 5 and 50 mW and spectrometer slit width was 300 µm. The scattered radiation was analysed by a SPEX Industries 1477B Triplemate spectrometer equipped with a SPEX Micramate microsampling system based on a modified Zeiss microscope. Diode array integration was used to obtain the spectra. Integration times on the diode array ranged from 2 to 30 s for 10 to 20 collections. Scattered radiation from the LDEF sample was collected by backscattering from the opaque material.

Murchison matrix. Fullerene particles between 0.05 and 0.1 mm in diameter were launched towards an aluminium plate at a velocity of 6.1 km s⁻¹. LIMS analyses of the crater residues indicate that most of the fullerenes did in fact survive impact. However, the impact velocities available from the two-stage light-gas gun are at the lower end of the velocity range for extraterrestrial particles striking LDEF at the position of crater no. 31. The mean encounter velocity of natural particles for this LDEF position is ~19 km s⁻¹, and the lower end of the velocity range may extend down to a few kilometres per second (ref. 13).

The origin of the fullerenes in crater no. 31 is unclear. If they are orbital debris, then their presence with chondritic material is

TABLE 1 Chemical analysis of crater no. 31 and clean Al plate

Element	Crater no. 31 (wt%)	Al plate* (wt%)
Si	1.8 (0.5–2.4)†	0.3
Mg	3.8 (1.1–4.6)	0.9
Ca	0.7 (0.1–0.95)	<0.02
Fe	0.3 (0.05–0.45)	<0.03
S‡	0.4 (0.5–0.65)	<0.02

All analyses represent averages of the wall and rim of the crater. (Al is omitted from the analyses.) The values for the crater represent the mean of 40 analyses; the plate values are the mean of 10 analyses. In order to obtain a flat surface, which is necessary for microprobe analyses, the raised rim and walls of the crater were gently pressed flat by using a hydraulic press and an Al₂O₃ disk (see Fig. 2*b*). Elemental analyses were performed by a CAMECA MBX scanning electron microprobe operated at 10 kV and 10 nA, with on-line PAP data corrections.

* A separate piece of the Al panel was polished and analyzed for background.

† Numbers in parentheses indicate analytical range.

‡ Sulphur is a known contaminant on LDEF surfaces and may not be a component of the object that caused the crater. Ni, Na, K, Cr, Mn, Ti were sought but not found above 0.03%.

fortuitous, although the probability is low for a micrometeoroid crater to be superimposed by a second crater made by orbital debris fullerenes. Moreover, there is no morphological evidence to support two cratering events. Production of fullerenes from an orbital debris carbon precursor on impact could have occurred at some velocity above $\sim 6 \text{ km s}^{-1}$ (our experimental limit) and less than $\sim 13 \text{ km s}^{-1}$, above which residue of the impactor is probably totally ejected (F. Hörz, personal communication). The nominal encounter velocity of circular orbital debris is $\sim 11 \text{ km s}^{-1}$ (see refs 13, 14). Thus, if the fullerenes did form in this manner, they probably did so at velocities near $9\text{--}13 \text{ km s}^{-1}$. Ideally, one would like to extend the hypervelocity experiments

to these higher velocities, although no particle accelerator exists that can meet the necessary requirements.

The fact that crater no. 31 contains chondritic elements suggests that the impactor was a micrometeoroid, but we do not accurately know its composition. The simplest explanation of the origin of the fullerenes is that they were either a component of a micrometeoroid and survived impact, or they were formed on impact from other carbonaceous material. The observation that fullerenes appear not to form in hydrogen-rich environments ($\geq 10\% \text{ H}_2$) limits the environments in which these fullerenes could have originated⁶. In any case, the results presented here are the first direct observation of fullerenes in space. $\square$

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1. Kroto, H. W., Heath, J., O'Brien, S. C., Curl, R. F. & Smalley, R. E. *Nature* **318**, 33–35 (1985).
2. Daly, C. F., Buseck, P. R., Williams, P. & Lewis, C. F. *Science* **259**, 1599–1601 (1993).
3. Buseck, P., Tsipursky, S. J. & Hettich, R. *Science* **257**, 215–217 (1992).
4. Krättschmer, W., Lamb, L. D., Fostiropoulos, K. & Huffman, D. R. *Nature* **347**, 354–355 (1990).
5. Gilmour, L. et al. *Lunar and Planetary Science* **XXII**, 445–447 (1991).
6. deVries, M. S., et al. *Geochim. cosmochim. Acta* **57**, 933–938 (1993).
7. Hörz, F. et al. in *Proc. 1st Long Deployment Exposure Facility Portretrieval Symp.* (ed. Levine, A.) 487–501 (NASA Conf. Proc. CP-3134, US Govt Print. Off., Washington DC, 1992).
8. Bunch, T. E. et al. in *Proc. 1st Long Deployment Exposure Facility Portretrieval Symp.* (ed. Levine, A.) 549–565 (NASA Conf. Proc. CP-3134, US Govt Print. Off., Washington DC, 1992).
9. Bunch, T. E. et al. in *Proc. 2nd Long Deployment Exposure Facility Portretrieval Symp.* (ed. Levine, A.) 453–477 (NASA Conf. Proc. CP-3194, US Govt Print. Off., Washington DC, 1993).
10. Brownlee, D. E. et al. in *Proc. 2nd Long Deployment Exposure Facility Portretrieval Symp.* (ed. Levine, A.) 577–584 (NASA Conf. Proc. CP-3194, US Govt Print. Off., Washington DC, 1993).

11. HcEvany, S. H. & Ross, H. H. *J. Am. Soc. Mass Spectrom.* **3**, 268–280 (1992).
12. Bethune, D. S. et al. *Chem. Phys. Lett.* **174**, 219–222 (1990).
13. Kessler, D. J. *J. Spacecraft* **28**, 347–351 (1991).
14. Coombs, C. R. et al. in *Proc. 2nd Long Deployment Exposure Facility Portretrieval Symp.* (ed. Levine, A.) 595–618 (NASA Conf. Proc. CP-3194, US Govt Print. Off., Washington DC, 1993).
15. Odom, R. W. & Schueler, B. in *Lasers and Mass Spectrometry* (ed. Lubman, D. M.) 103–137 (Oxford Univ. Press, New York, 1990).
16. Weeks, S., D'Silva, A. P. & Dobson, R. L. M. in *Lasers and Mass Spectrometry* (ed. Lubman, D. M.) 510–529 (Oxford Univ. Press, New York, 1990).
17. O'Neal, R. L. & Lightner, E. B. in *Proc. 1st Long Deployment Exposure Facility Portretrieval Symp.* (ed. Levine, A.) 3–48 (NASA Conf. Proc. CP-3134, US Govt Print. Off., Washington DC, 1992).

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Near-field spectroscopy of single molecules at room temperature

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THE ability to observe the optical spectrum of a single molecule can afford insights into the interactions that distinguish one molecular environment from another. Such sensitivity has recently been achieved at liquid-helium temperatures^{1–5}. Here we show that the near-field scanning optical microscope^{6,7} can be used to obtain the time-dependent emission spectrum of a single molecule in air at room temperature, with a spatial resolution of about 100 nm. We have examined single molecules of 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine (diI) dispersed on polymethylmethacrylate. The spectra of individual molecules exhibit shifts of $\pm 8 \text{ nm}$ relative to the average spectrum, and are typically narrower, as is expected for spectral lines broadened inhomogeneously (that is, by a distribution of molecular environments). The spectra also vary in width by up to 8 nm, some being as broad as the far-field many-molecule spectrum. The emission spectra of some individual molecules exhibit time-dependent shifts of up to 10 nm. This variety in spectral position, width, shape and time dependence can be understood within a model of inhomogeneous broadening in which there is a distribution of barrier heights to rearrangement of the molecular environment.

In Fig. 1 are shown a schematic view of the experiment and a near-field fluorescence image of individual diI molecules (Molecular Probes) on a thin film of polymethylmethacrylate (PMMA). As an imaging microscope, the present configuration is similar to that recently reported⁸ for observing single molecules. Spectra were obtained by first locating an isolated molecule in imaging mode, then switching the fluorescence emission from the spectrally integrating detector to a spectrograph (see Figure captions for experimental details).

Single-molecule spectra are shown in Figs 2 and 3. Taken together, these constitute a fairly representative set of the spec-

tral types observed. In addition to the two spectra displayed in Fig. 2, a near-field image of that pair of molecules is presented, demonstrating the spatial resolution of near-field spectroscopy. The spectrum of molecule B, which was acquired first, contains no measurable contribution from molecule A, although it lay only $\sim 150 \text{ nm}$ away. This is supported by the fact that when B photobleached, the spectrum dropped to the background level. In Fig. 3 there are two spectra, labelled D and E, displaying different intensities in the vibronic band, and a spectrum labelled C, that is broadened. Twenty-eight single-molecule spectra, acquired under non-perturbing conditions (see caption, Fig. 1b) were classified by the wavelength of the emission maximum, bandshape and temporal invariance. Each of the various bandshapes could be adequately represented by one of two basis spectra, or one of the functions given by the convolution of those spectra with gaussian distributions. The two basis spectra were D and E. Overlaid with spectrum C is the convolution of D with a 15-nm full width at half maximum (FWHM) gaussian, shifted to shorter wavelength by 5 nm. The spectra in Fig. 2 have relatively higher oscillator strength in the vibronic band, and so are better represented by the convolution of E with an 8-nm gaussian. Half the single-molecule spectra could be represented by the basis functions alone, and half required convolution using gaussians having widths up to 15 nm. The far-field, many-molecule spectrum is reproduced by a combination of the basis functions convolved with a 15-nm gaussian.

In addition to the spectral variations among molecules, the emission spectra of about 20% of the individual molecules were observed to vary in time, as seen previously in ref. 9. An example is given in Fig. 4. After 17 minutes during which the spectrum was invariant, it red-shifted 5 nm during the eighteenth minute and bleached the following minute. More data is required before concluding that the spectral shifts and the photobleaching probability are connected.

The simplest model of inhomogeneous broadening consists of a basis spectrum convolved with a distribution function of transition energies. Although 28 molecules are hardly sufficient to define a distribution, a gaussian fit to the histogram of emission maxima yields the same 15-nm width parameter that was required to fit the far-field spectrum in the manner discussed above. A characterization of the interactions producing the shifts is beyond the present work. Suffice it to say that diI has a small

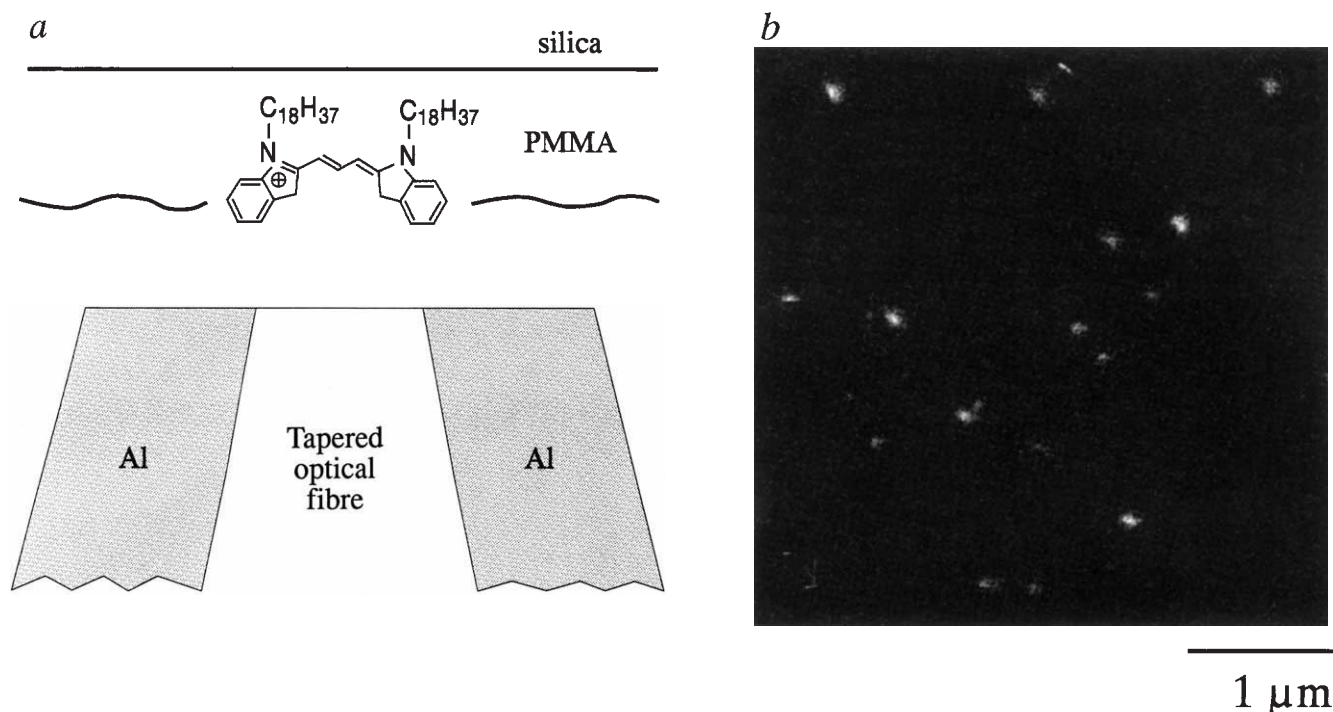


FIG. 1 *a*, Schematic diagram of near-field spectroscopy. An aluminium-coated, tapered optical-fibre probe¹² spatially confines 529-nm excitation light to ~ 100 -nm (the aperture diameter of the probe tip). The probe is maintained ~ 10 nm above the surface of a <15 -nm-thick film of polymethylmethacrylate (PMMA), on which dil molecules have been dispersed at an average coverage of $<10 \mu\text{m}^{-2}$. Fluorescence is collected through the silica coverslip by an NA 1.4 microscope objective (not shown), and detected by either a low-noise avalanche-photodiode detector (not shown), or dispersed through a 0.27-m monochromator onto cooled CCD (charge-coupled device) camera for single-molecule spectroscopy. The samples were prepared by spin-coating the coverslip with a drop of a dilute solution of PMMA in toluene, followed by a drop of $\sim 3 \times 10^{-9}$ M solution of dil in heptane. The experimentally determined fluorescence quantum yield was near unity. The photobleaching branching ratio, defined as the probability per photoexcitation that a molecule undergoes a photochemical modification that permanently quenches its fluorescence, was found to be highly nonexponential, with an initial value of $\sim 10^{-6}$ – 10^{-7} and a value of $\sim 10^{-8}$ after ~ 80 – 90%

of the molecules had been bleached. *b*, Near-field-excited fluorescence image of single dil molecules on a PMMA film. This image was acquired under the same conditions as the majority of the single-molecule spectra. The excitation power was ~ 10 nW, resulting from coupling $50 \mu\text{W}$ into the single-mode fibre and an aperture transmission coefficient of 2×10^{-4} ; the probe-sample distance was 9 ± 1 nm, regulated by a shear-force feedback^{13,14} loop utilizing a tip dither of ~ 8 nm. Restricting the dither amplitude to ~ 10 nm, while maintaining a vertical stability of ± 1 nm at a probe-sample distance of >5 nm, was necessary to ensure that the near-field probe did not physically perturb the molecular environment. The power in the probe was maintained at or below ~ 0.1 mW so as to minimize the heating of the probe due to absorption by the aluminium coating and the consequent sample heating. Under these conditions, $>50\%$ the molecules emitted the $\geq 3 \times 10^6$ photons necessary to obtain a near-field spectrum having sufficiently high signal-to-noise ratio for the degree of spectral analysis applied. The image acquisition time for the 250×250 pixel frame displayed here was ~ 15 min.

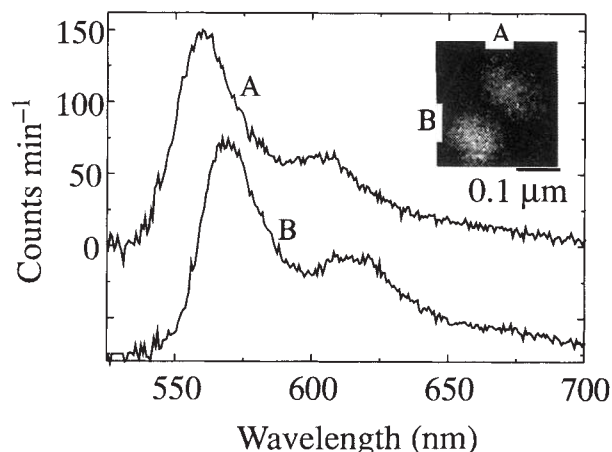
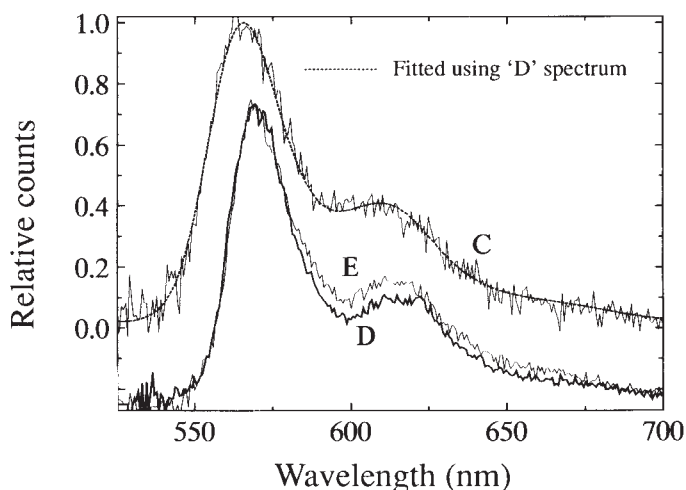


FIG. 2 The near-field fluorescence image of two adjacent molecules A and B (see inset) and the corresponding fluorescence spectrum of each. The data were obtained as follows. First, the image was acquired, then the fibre probe was moved over molecule B where it was held stationary while collecting, dispersing and integrating the fluorescence in 1-min intervals until photochemical degradation extinguished the emission. The resulting spectrum is shown as curve B. The sample area was then re-imaged revealing only molecule A, and the fibre probe was then moved over molecule A where the spectrum labelled A was measured in the same manner. With the probe held stationary over a single molecule, there is no measurable excitation of another molecule lying 150 nm away as evidenced by the spectra displayed here, and even more strongly by the fact that the fluorescence signal goes to zero when the molecule under the aperture photobleaches. The displayed spectrum B is the average of 21 1-min integrations, and lies near the centre of the distribution of fluorescence wavelengths. Spectrum A is the average of five 1-min integrations, and lies on the high-energy side of that distribution, being shifted ~ 10 nm to the blue. Each 'count' on the y-axis of the spectrum is the result of ~ 200 photons emitted by the molecule. Spectrum B is displayed vertically displaced for clarity.

FIG. 3 Spectra of two molecules, D and E, exhibiting different intensities in the vibronic band, and of one, C, having a broadened spectrum. The fit to the spectrum of molecule C is the convolution of D with a 15-nm gaussian (FWHM), shifted to shorter wavelength by 5 nm. Although molecule C photobleached after a single 1-min integration, photostability and spectral width were generally uncorrelated. E was shifted 5.5 nm to longer wavelength to facilitate bandshape comparison, and D and E were displaced vertically for clarity.



change in dipole moment on optical excitation¹⁰, and so its fluorescence spectrum is not very sensitive to solvent polarity. It is, however, fairly polarizable. Therefore, variations in the local polarizability are probably dominant in producing the inhomogeneous width.

In going beyond simple inhomogeneous broadening models, one must take into account the variability in the spectral width of individual molecules. Our interpretation of this phenomenon is that those molecules having narrow spectra sit in rigid environments. Those having broader spectra exist in more dynamic surroundings in which molecular reorientation of the matrix molecules, and the concomitant shift in the emission wavelength, occurs many times within the 1-min spectral integration. Spec-

trum C in Fig. 3 is one of the broadest, for which the fluctuations in the molecule-matrix interaction energy inferred from the width of the gaussian used in the fit is $\sim 2kT$ (kT being the thermal energy). We also interpret the 'discrete' spectral jumps within this scheme. In this case, changes in the local environment occur on a timescale of minutes rather than seconds. Thus, the activation energy for the rearrangement must be $\sim 4kT$ higher. An implication of the model is that the width of a single-molecule spectrum will depend on the timescale of the measurement. This is closely related to the effect, demonstrated a few years ago¹¹, that the 'homogeneous' width of a molecular transition in an organic glass at low temperature depends on the timescale of the measurement. Finally, we note that we found no correlation between the various bandshapes and the spectral shift, nor between the strength of the vibronic feature and the spectral width.

A concern with a new technique, such as near-field spectroscopy, is that the measurement perturbs the sample sufficiently to influence the observed phenomena. Our evidence against this is summarized here. Operating under the conditions detailed in the caption to Fig. 1b, the near-field spectra sum to give the far-field spectrum, the photochemical stability is consistent with far-field measurements, and neither the probability of observing a spectral jump nor a broadened spectrum was altered by changing the feedback conditions or the laser power.

To understand better the site inhomogeneity and the broadening processes, the present apparatus can be adapted to perform simultaneous fluorescence lifetime and emission measurements, and to operate variably between low and room temperatures. Additionally, it may be used to study a single molecule in a well-characterized molecular environment, such as a protein binding pocket, where the primary interest is under physiological conditions, and to do so even if the sites of interest are only ~ 100 nm apart. □

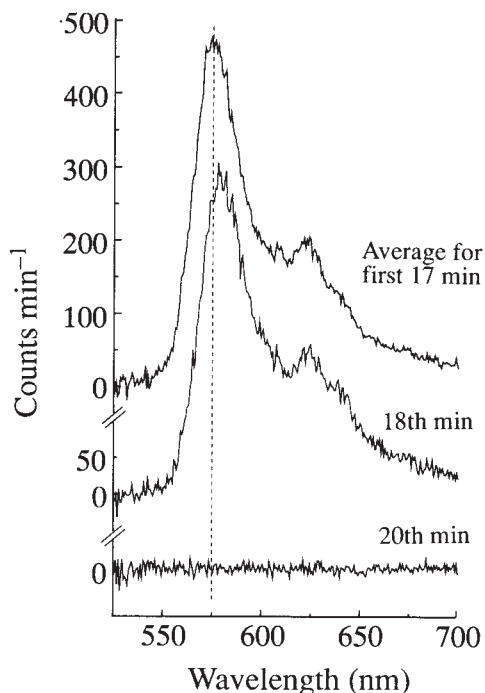


FIG. 4 Single-molecule spectra of a highly photostable molecule. The upper trace is the average of 17 spectra acquired over 17 min during which no distinguishable spectral change occurred. During the eighteenth 1-min integration, the emission red-shifted ~ 5 nm. In the nineteenth minute, the molecule photobleached. The lowest trace (labelled twentieth minute) is the background. We calculate that $\sim 2 \times 10^8$ photons were emitted by this molecule during the spectral integration.

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- Moerner, W. E. & Kador, L. *Phys. Rev. Lett.* **62**, 2535-2538 (1989).
- Orrit, M. & Bernard, J. *Phys. Rev. Lett.* **65**, 2716-2719 (1990).
- Tchéniou, P., Myers, A. B. & Moerner, W. E. *Chem. Phys. Lett.* **213**, 325-332 (1993).
- Moerner, W. E. & Basché, T. *Angew. Chem. int. Edn. engl.* **32**, 457-476 (1993).
- Orrit, M., Bernard, J. & Personov, R. I. *J. phys. Chem.* **97**, 10256-10268 (1993).
- Betzig, E. & Trautman, J. K. *Science* **257**, 189-195 (1992).
- Pohl, D. W. & Courjon, D. (eds) *Near Field Optics* (Kluwer, Dordrecht, 1993).
- Betzig, E. & Chichester, R. J. *Science* **262**, 1422-1425 (1993).
- Ambrose, W. P. & Moerner, W. E. *Nature* **349**, 225-227 (1991).
- Orrit, M. et al. *Chem. Phys. Lett.* **129**, 232-236 (1991).
- Berg, M., Walsh, C. A., Narasimhan, L. R., Littau, K. A. & Fayer, M. D. *Chem. Phys. Lett.* **139**, 66-71 (1987).
- Betzig, E., Trautman, J. K., Harris, T. D., Weiner, J. S. & Kostelak, R. L. *Science* **251**, 1468-1470 (1991).
- Betzig, E., Finn, P. L. & Weiner, J. S. *Appl. Phys. Lett.* **60**, 2484-2487 (1992).
- Toledo-Crow, R., Yang, P. C., Chen, Y. & Vaez-iravani, M. *Appl. Phys. Lett.* **60**, 2957-2960 (1992).

Nitride glasses obtained by high-pressure synthesis

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THE incorporation of nitrogen in oxide glasses can lead to improved physical properties such as hardness and refractive index¹⁻⁴; one might accordingly expect to see a further improvement in glasses containing nitrogen as the only anion. But while there are now many examples of non-oxide glasses in the halide and chalcogenide families⁵⁻⁸, the formation of pure nitride glasses has not hitherto been demonstrated. Here we report the formation of glasses in the system $\text{Li}_3\text{N}-\text{Ca}_3\text{N}_2-\text{P}_3\text{N}_5$, by the rapid quenching of fused mixtures of the nitrides at high pressures. The use of high-pressure conditions prevents thermal decomposition of the nitride mixtures to gaseous nitrogen. The new nitride glasses are stable in air, have remarkably high refractive indices (1.97–2.0), hardness exceeding that of silica glass, and high glass transition temperatures ($T_g > 700^\circ\text{C}$).

The preparation and properties of oxynitride glasses have recently been reviewed¹⁻⁴. The syntheses of such glasses have been performed either by quenching fused mixtures of oxides and nitrides¹⁻⁴, or by reacting NH_3 with molten oxides⁹. Under these conditions, the nitrogen content has been limited by the decomposition of melts to form gaseous nitrogen and in some cases formation of nitride crystals. No more than one of the four oxygen atoms surrounding the glass-forming cation (Si^{6+} , P^{5+} , Al^{3+}) has been replaced by nitrogen in silicate and phosphate glasses prepared by these two synthetic routes. These studies have demonstrated that the physical properties are improved when nitrogen is incorporated in the glasses. The hardness, refractive index, chemical durability, glass transition temperature and softening temperature all increase significantly with increasing nitrogen content, while the thermal expansion coefficient decreases^{4,9}. These changes in the physical properties are consistent with a structural model in which divalent oxygen is substituted by trivalent nitrogen to produce a more tightly linked glass network¹⁻⁴. Clearly the properties of glasses with only N^{3-} anions would be of interest.

To obtain nitride glasses from the melt, the first requirement is that the melt be stable against decomposition, achieved in our study by application of high external pressure and enclosure of the sample in a sealed container¹⁰. This renders any potential decomposition reaction thermodynamically unfavourable, and offers fast cooling rates (100 K s^{-1}), which is important to suppress crystallization. Next, a glass-forming composition must be found. Typical glass-forming melts in oxide systems contain the network-forming species Si^{4+} , B^{3+} , Al^{3+} or P^{5+} . The first of these form stable crystalline nitrides¹¹, but these are so refractory¹² that the viscosities of their melts would be very low at their freezing temperatures: vitrification would therefore require hyperquenching. In contrast, P_3N_5 typically forms an amorphous powder, which is very difficult to convert to a dense crystalline form¹³⁻¹⁵. This would appear to be a good starting component for a class of glasses based on a phosphorous-nitrogen network. In oxide glass chemistry, it is usual to add 'modifier' components to the network component to expand the glass-forming domain, accompanied by network bond-breaking (depolymerization). In our study, we focused on the system $\text{Li}_3\text{N}-\text{P}_3\text{N}_5$. Li_3N is one of the most stable molten nitrides, and has a relatively low melting point (813°C)¹². In addition, the

tetrahedral PN_4 network found in LiPN_2 is structurally analogous to that of SiO_2 cristobalite¹³, itself an excellent glass former. Several other crystal structures based on linked PN_4 tetrahedra are known in the $\text{Li}_3\text{N}-\text{P}_3\text{N}_5$ system¹³⁻¹⁶. The compounds, with different N:P ratios, can be described in terms of decreasing polymerization of the tetrahedral PN_2 -network found in LiPN_2 , analogous to the case of glass-forming binary silicates. In addition, some of the highest nitrogen contents in oxynitride glasses to date have been found in the $\text{Li}-\text{P}-\text{O}-\text{N}$ system^{9,17}. It is also common in glass chemistry to add a third component, thereby enhancing glass formation by increasing the entropy of the mixture and decreasing the liquidus temperature. For our nitride glass syntheses we chose Ca_3N_2 , also a stable nitride with a relatively low melting point ($1,195^\circ\text{C}$)¹², as a third (network modifying) component.

In Fig. 1 we show the glass-formation field in the system $\text{Li}_3\text{N}-\text{Ca}_3\text{N}_2-\text{P}_3\text{N}_5$. The glasses were obtained by an isobaric quench at 8 kbar from $1,400^\circ\text{C}$ to room temperature. The samples were examined by polarized optical microscopy, scanning electron microscopy, electron microprobe analysis (both wavelength and energy dispersive modes), X-ray powder diffraction and micro-infrared and Raman spectroscopy. The glassy nature of the

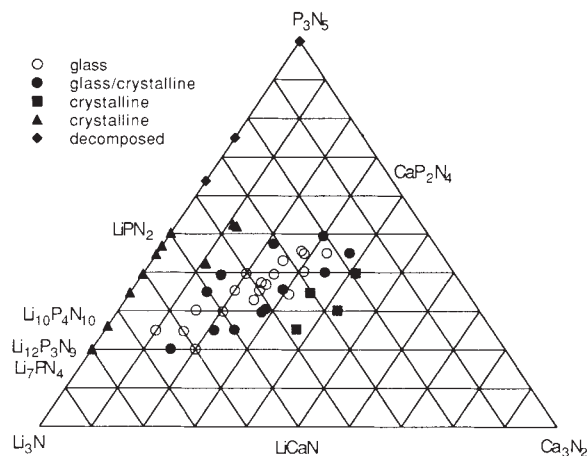
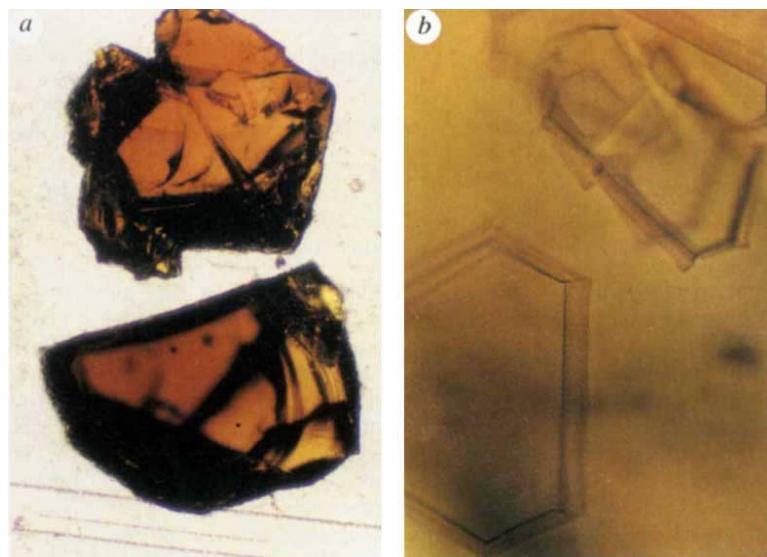


FIG. 1 The glass-formation area in the ternary system $\text{Li}_3\text{N}-\text{Ca}_3\text{N}_2-\text{P}_3\text{N}_5$ at 8 kbar and $1,400^\circ\text{C}$. Ground mixtures of the starting materials (Johnson Matthey) were compressed into cylindrical capsules of tantalum (molybdenum) in a glove box. The capsule was closed by a Ta(Mo)-cap tightly fitting the capsule and then placed in a platinum capsule which was sealed by arc-welding. The Pt-capsule prevented contamination and degassing of the sample while the Ta(Mo) capsule protected the platinum from corrosion by the molten nitrides. The experimental assembly for the piston-cylinder runs is described by Holloway¹⁰. The samples were preheated to $1,000^\circ\text{C}$ and 10 kbar for 30 min to enhance a reaction between the starting materials before melting the sample. The assembly was finally heated to $1,400^\circ\text{C}$ at 8 kbar for 20 min and isobarically quenched to room temperature at a cooling rate of $\sim 100\text{ K s}^{-1}$ between 500 and $1,400^\circ\text{C}$. The samples marked with triangles and diamonds were quenched from higher pressures in the region 10–20 kbar and varying temperatures in the region $1,100$ – $1,500^\circ\text{C}$. These were the initial experiments carried out to search for the best glass-forming conditions. Samples with $>50\text{ mol}\%$ P_3N_5 quenched from $1,500^\circ\text{C}$ were found to have decomposed to a supercritical fluid N_2 phase (retained in the capsule) and elemental P. All later runs used $1,400^\circ\text{C}$ and 8 kbar. Samples with $>90\text{ vol}\%$ glass are shown as open circles and samples with $\geq 90\text{ vol}\%$ crystalline material are given as filled squares in the Figure. Samples containing roughly equal amounts of glass and crystal are shown as filled circles. The compositions of 10 glass samples were confirmed by electron microprobe analysis (wavelength and energy dispersive modes), and further supported in one case by Rutherford back scattering. Up to 1.5 at% Mo or Ta was found in some, but not all, of the samples. No oxygen was found in the samples, within analytical error ($< 1\text{--}2\%$). The glasses were found to be stable in air.

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FIG. 2 Examples of a fully vitrified sample (a) and a partly crystalline sample (b). The fragments of the glass shown are ~0.1–1.0 mm diameter. The sizes of the crystals that were found in some samples (for example, b) were observed to vary systematically with composition. Large crystals (100–300 μm), evidently present before the isobaric quench, were observed in samples with 0.45 mol% $\text{P}_3\text{N}_5 > 0.2$. Crystals two orders of magnitude smaller were observed in samples with high Li_3N or Ca_3N_2 content. Samples with >40 mol% P_3N_5 showed a tendency to decompose, evidenced by a dark colour. The glass-formation field is therefore probably only intrinsically limited by crystallization on the Li_3N side. The observation of glass formation in the presence of primary crystals (b) implies that crystal growth rates are slow, hence the melt was very viscous at the quenching temperature, 1,400 $^\circ\text{C}$. Conchoidal fractures and bubbles, typically found in glassy materials, are easily seen in the glassy samples. The high refractive index of the glass is indicated by the very dark outlines in these photographs. The small bubbles correspond to some exsolved nitrogen. (Magnifications: a, $\times 24.5$; b, $\times 196$.)



samples was verified by the isotropic properties under polarized light, the lack of sharp X-ray diffraction peaks, and the appearance of broad vibrational bands in the infrared and Raman spectra.

The intrinsic colour of the glasses is yellow, turning slightly red with increasing Li_3N content. A shade of green was observed in some of the Ca_3N_2 -rich samples with high N:P ratios. Photographs of a typical sample of the nitride glass and a partly crystalline sample are shown in Fig. 2. Conchoidal fractures and bubbles, typically found in glassy materials, are easily seen in the glassy samples. The bubbles correspond to exsolved nitrogen. However, most of the nitrogen is contained in the bulk glass matrix, as verified by the chemical composition of the glasses and their vibrational spectra.

Fundamental to glasses is the transition at the temperature T_g from glass to a supercooled liquid on heating. We tried to observe this by hot-stage microscopy and differential thermal analysis (DTA). Due to small sample size relative to the DTA assembly and the unexpected weakness of the transition, T_g was not clearly observed. However, X-ray diffraction confirmed crystallization of glasses heated to 900 $^\circ\text{C}$, whereas heating to 700 $^\circ\text{C}$ did not show any indication of devitrification. A strong exothermic crystallization (onset 800 $^\circ\text{C}$) was recorded for one of the samples with high content of Li_3N . Viscous flow was observed to occur between 750 and 850 $^\circ\text{C}$, but the glass powders were not observed to fuse together strongly below 900 $^\circ\text{C}$. Small

heat-capacity changes at T_g and a weak tendency for viscous flow are typical of systems like SiO_2 and BeF_2 , and indicate a 'strong' liquid behaviour of these nitrides, which is unexpected because the N:P ratio is higher than in the ideal, fully-polymerized, tetrahedral network.

The thermal decomposition of the samples resulted in a much larger and more easily observed endothermic event. The decomposition temperature did not change significantly with composition, with an onset temperature around 930–1,000 $^\circ\text{C}$, and full decomposition at 1,080–1,130 $^\circ\text{C}$.

The glasses reported here probably have a structure involving partly polymerized PN_4 -tetrahedra. Such a model is based on the structures of the known crystalline compounds in the Li-P-N system; these include isolated PN_4^{7-} anions in Li_7PN_4 (ref. 14), partly polymerized PN_4 -tetrahedra as $\text{P}_4\text{N}_{10}^{10-}$ and $\text{P}_3\text{N}_9^{12-}$ anions in $\text{Li}_{10}\text{P}_4\text{N}_{10}$ and $\text{Li}_{12}\text{P}_3\text{N}_9$ (refs 15, 16) and a fully polymerized PN_2 -tetrahedral network in LiPN_2 (ref. 13). A partly polymerized tetrahedral glass structure is consistent with the infrared and Raman spectra of our glasses, in which bands due to stretching and bending vibrations of PN_4 tetrahedral groups can be recognized (Fig. 3). Spectroscopic studies of glasses in the Li-P-O-N system also support a structure based on partly polymerized $\text{P}(\text{O}, \text{N})_4$ tetrahedra¹⁷.

The sharply defined glass-formation field lies at surprisingly high contents of Li_3N and Ca_3N_2 ; the concentration of P_3N_5 in the glasses is lower than originally expected. The N:P ratio in

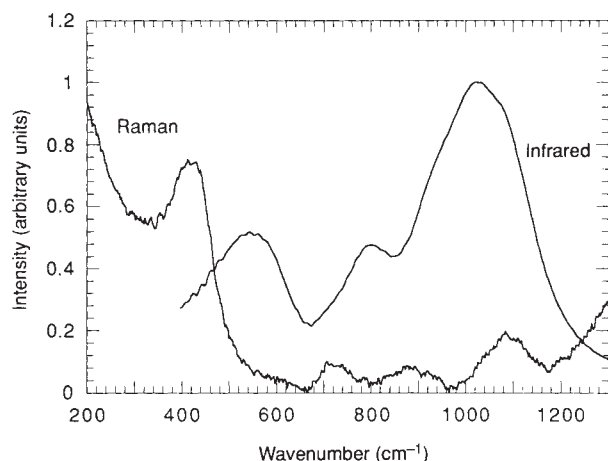


FIG. 3 Raman and infrared spectra of a typical nitride glass with composition 40.0 mol% Li_3N , 24.8 mol% Ca_3N_2 and 35.2 mol% P_3N_5 . The increasing intensity in the Raman spectra above 1,200 cm^{-1} is due to a fluorescence band, probably due to Ta contamination. The bands in the 700–1,100 cm^{-1} region are due to P–N–P stretching vibrations, whereas those at lower frequency are associated with N–P–N and P–N–P bending¹⁷. More detailed assignments will be possible after further study of model compounds.

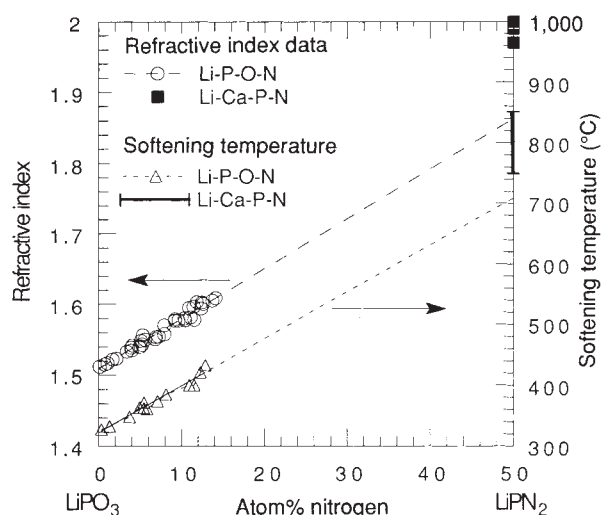


FIG. 4 Refractive indices and softening temperatures of the nitride glasses compared to known and extrapolated values of glasses in the system Li-P-O-N (ref. 9). The extrapolated lines for the Li-P-O-N glasses correspond to a linear change in composition going from LiPO_3 to LiPN_2 . The refractive indices of the nitride glasses were measured by the immersion technique. The extrapolated values for LiPN_2 glasses fall below the values measured for the $\text{Li}_3\text{N}-\text{Ca}_3\text{N}_2-\text{P}_3\text{N}_5$ glasses, implying an important role for the Ca_3N_2 component. The $\text{Li}_3\text{N}-\text{Ca}_3\text{N}_2-\text{P}_3\text{N}_5$ glasses also have a higher N:P ratio than that in LiPN_2 .

the glasses remains around 2.6 ± 0.6 , which corresponds to a degree of polymerization intermediate between one-dimensional chains and rings (N:P=3.0) and a nearly fully polymerized tetrahedral network (N:P=2.0).

Refractive indices of the nitride glasses, which are typically in the range 1.97–2.0, are shown in Fig. 4 together with corresponding data for Li-P-O-N glasses along the join LiPO_3 – LiPN_2 . A similar comparison of the softening temperatures of the oxynitride and nitride glasses is also shown in Fig. 4. The hardness of the nitride glasses was estimated by a scratching test using vitreous silica and quartz (SiO_2) (5.5 and 7 on Mohs' scale). The nitride glasses were found to be harder than vitreous silica (but softer than quartz), hence much harder than the corresponding metaphosphate glasses and consistent with the inferred high T_g . The Li^+ ion conductivity might also be of interest as indicated by the high Li^+ conductivity observed in Li_3N , Li_7PN_4 and $\text{Li}_{10}\text{P}_4\text{N}_{10}$ (refs 18, 19) although glasses with high T_g usually have low ionic conductivities at room temperature.

We expect that the range of glass-forming compositions can be greatly expanded from the base system we describe here, and that the combination of high hardness and high refractive index will make this new class of inorganic glasses valuable for specialized technological purposes, for example, in coupling to high-density crystalline materials in optical devices. The present high-pressure synthesis is probably not practical for bulk glass preparation. However our finding of a composition range for formation of glasses, which are thermally stable at their ambient-pressure glass transition temperatures suggest that glasses with the desirable properties we have described should be obtainable by ambient-pressure synthetic routes, such as those based on the sol-gel approach.

Note added in proof: We have now prepared a Ca-free glass, with approximate composition Li_4PN_3 . □

- Rawson, H. *Inorganic Glass-Forming Systems* (Academic, New York, 1967).
- Zarzycki, J. *J. Glasses and the Vitreous State* (Cambridge Univ. Press, 1990).
- Larson, R. W. & Day, D. E. *J. non-cryst. Solids* **88**, 97–113 (1986).
- Hollaway, J. R. & Wood, B. J. *Simulating the Earth; Experimental Geochemistry* (Unwin Hyman, Boston, 1988).
- Wells, A. F. *Structural Inorganic Chemistry* (Clarendon Press, Oxford, 1984).
- Chase, M. W. et al. *Janaf Thermochemical Tables 3rd edn* (American Chemical Soc., Washington DC, 1985).
- Schnick, W. & Luecke, J. Z. *anorg. allg. Chem.* **588**, 19–25 (1990).
- Schnick, W. & Luecke, J. *J. Solid St. Chem.* **87**, 101–106 (1990).
- Schnick, W. *Angew. Chem., int. Edn Engl.* **32**, 806–807 (1993).
- Schnick, W. & Berger, U. *Angew. Chem., int. Edn engl.* **30**, 830–832 (1991).
- Bunker, B. C. et al. *J. Am. Ceram. Soc.* **70**, 675–681 (1987).
- Schnick, W. & Luecke, J. *Solid St. Ionics* **38**, 271–273 (1990).
- Rabenau, A. *Solid St. Ionics* **6**, 277–293 (1982).

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Effect of variations in supersaturation on the formation of cloud condensation nuclei

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SULPHATE aerosols can act as nuclei for cloud formation, thereby cooling the climate by increasing the Earth's albedo^{1–5}; however the magnitude of this effect is very uncertain^{3,6}. Recently, Langner *et al.*⁷ calculated that at most 6% of the anthropogenic sulphur emission forms new particles, while 44% adds mass to existing sulphate particles activated in clouds. It was therefore suggested^{7,8} that previous studies^{1,2,9} had overestimated the effect of sulphate aerosols on climate. Although it has been proposed that sub-CCN-size particles can grow to CCN-size in clouds^{7,10}, this was thought to require the large supersaturations present in cumuliform clouds, rather than the smaller values characteristic of marine stratiform clouds, which are most important for radiative forcing. Here we show that natural variability of even low average supersaturations allows particles as small as $0.015 \mu\text{m}$ to grow to become CCN. This process can quadruple the CCN concentration and significantly increase the corresponding aerosol effect on climate.

The concentration of available CCNs depends on the cloud supersaturation (S_c), the aerosol size distribution and their chemical composition. A given cloud supersaturation activates (transforms into a cloud drop) hygroscopic particles larger than a critical radius¹¹, r_c (where $r_c (\mu\text{m}) = 0.0145 - S_c^{-2/3}$). The cloud droplet can then take up gaseous SO_2 which is subsequently oxidized. When the cloud droplet evaporates, the additional sulphate generated by this process is deposited on the CCN, increasing its size; although this mechanism does not generate new particles, it means that existing CCN can subsequently be activated in lower supersaturations. Such in-cloud modification of the aerosol size distribution has been seen in field measurements^{12–14} and theoretical analysis¹⁰.

Langner *et al.*⁷ estimated that 44% of anthropogenic SO_2 is absorbed by cloud droplets, and that only 6% of the anthropogenic SO_2 emissions is used to create new particles. Although they acknowledged that, in principle, in-cloud growth can modify the aerosol size distribution so that additional particles can be activated at a given supersaturation, they focused their discussion on the new particles and concluded that anthropogenic emissions have a much smaller effect on the CCN budget

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- Jack, K. H. *J. Mater. Sci.* **11**, 1135–1158 (1976).
- Loehman, R. E. *J. non-cryst. Solids* **42**, 433–446 (1980).
- Loehman, R. E. *J. non-cryst. Solids* **56**, 123–134 (1983).
- Sakka, S. A. *Rev. Mater. Sci.* **16**, 29 (1986).
- Lucas, J. *J. Mater. Sci.* **24**, 1–13 (1989).
- Poulain, M. *Nature* **293**, 279–280 (1981).

than estimated before². However, if the supersaturation was high even temporarily, small particles could be activated and then converted into larger particles by in-cloud processing. As a particle can go through 10 cloud cycles before removal by precipitation¹³, the occurrence of a high supersaturation value during any one of these cycles will increase the size of small particles that were not otherwise active as CCNs, and transform them into efficient CCNs in their remaining interactions with clouds.

Here, using a simple aerosol growth model, we calculate the contribution of SO_2 to the generation of new CCNs in the presence of variations in the cloud supersaturation. The model calculates the concentration of new CCNs as a function of the fraction of SO_2 oxidized to sulphate in gas phase, f_1 (that is, 6%), and σ_{sc} , the standard deviation of the supersaturation. Because half the SO_2 is deposited in dry deposition⁷, $0.5-f_1$ (that is, 44%) is oxidized in cloud droplets. It will be shown that even for the low average marine cloud supersaturations, the new

numerous CCNs added by variations in the supersaturation increase the equilibrium CCN concentration in the presence of deposition by precipitation.

The model describes the time evolution of the aerosol size distribution, initiated with a typical log-normal aerosol distribution present in the absence of cloud interactions¹⁵ (Fig. 1a). A size distribution of particles just formed from oxidation of SO_2 was also introduced to some of the calculations, but due to the small size of these particles and the fast coagulation rate¹³ it did not alter significantly the CCN distribution. At every one of the 100 time steps used in the calculations (covering the 6 days of the aerosol lifetime) a fixed fraction ($\Delta M_{sg} = f_1 M_s / 100$) of the initial SO_2 concentration, M_s , is converted into new particles that resemble the original size distribution. At every step a value of the supersaturation is randomly chosen, but so that an ensemble of the values forms a gaussian or boxcar probability distribution. Assuming that each particle cycles through clouds 10 times¹³, 10% of the particles are present in clouds in every time step. Out of this 10%, particles that are large enough to be activated for the chosen supersaturation gain in volume by absorbing the oxidized sulphate. They share $\Delta M_{sc} = (0.5-f_1) M_s / 10$ of the sulphur mass.

It is assumed that each cloud drop can oxidize the same amount of SO_2 (ref. 16), thus neglecting effects of drop size and acidity^{17,20}. This assumption requires some clarification. An

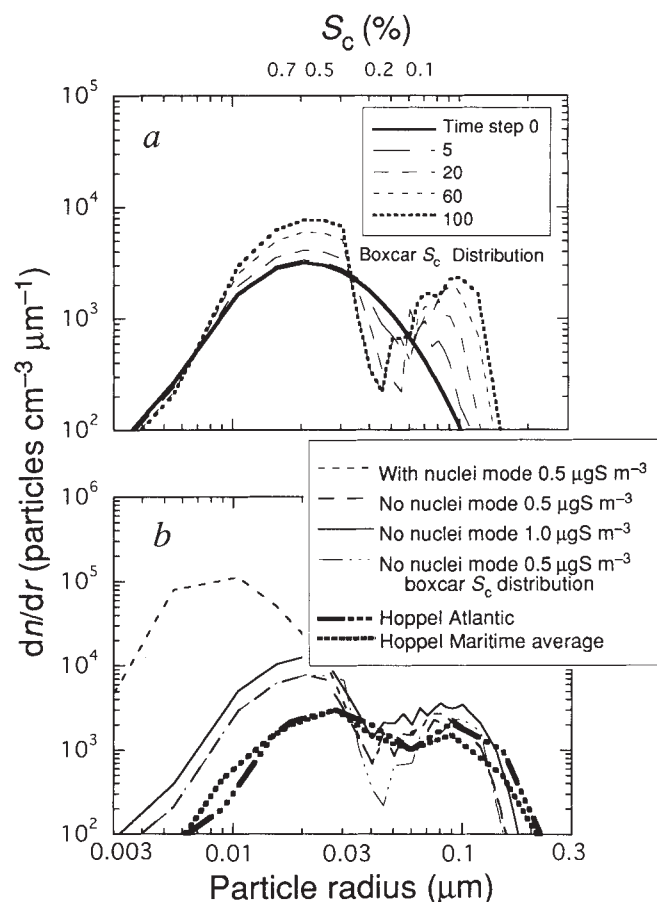


FIG. 1 Model calculations of the aerosol size distributions (dn/dr is the number of particles per interval radius) for maritime conditions with the following assumptions. Variable supersaturation $S_c = 0.2 \pm 0.1\%$; 12% of sulphur is oxidized to sulphate in the gas phase and 38% in the aqueous phase. The initial size distribution had 120 particles cm^{-3} . *a*, Time dependence of the aerosol size distribution with 100 time steps across the 6 days of aerosol lifetime; sulphur concentration of $M_s = 0.5 \mu\text{g-S m}^{-3}$. *b*, Final size distribution for four conditions: varying the value of M_s ; the presence of the nuclei mode (mode of particles just formed from sulphur oxidation in the gas phase); varying the shape of the probability distribution of the supersaturation between gaussian and boxcar. Measured size distributions are plotted in heavy dashed lines after Hoppel et al.¹³. The value of the supersaturation, S_c , is given at the top of the figure for two corresponding sizes of particles activated for this value of S_c .

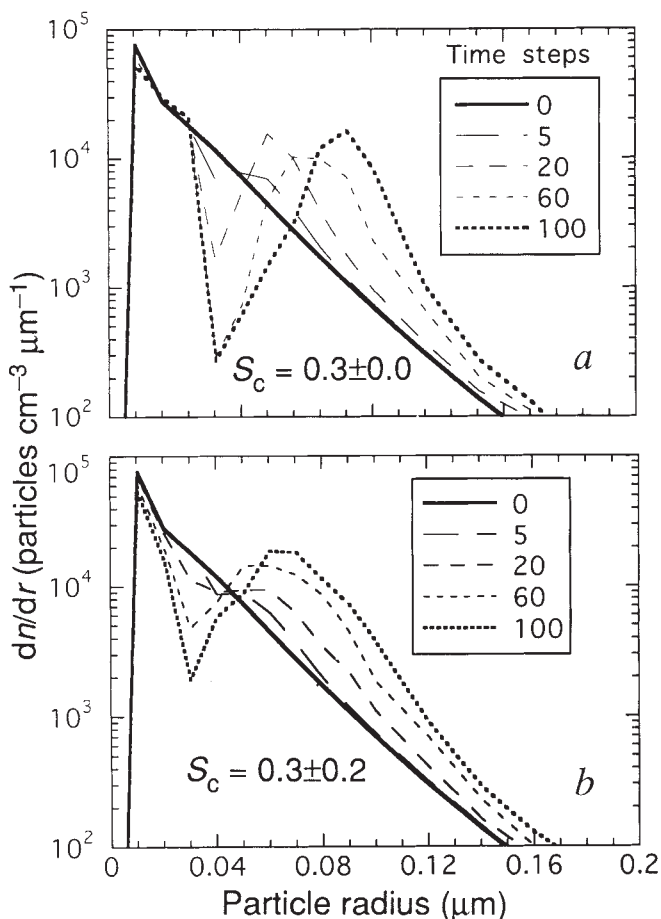


FIG. 2 The time dependence of the aerosol size distribution initiated by 800 particles cm^{-3} , given by the time step. The 100 time steps correspond to the aerosol lifetime of 6 days. M_s , the initial SO_2 concentration, is $1 \mu\text{g-S m}^{-3}$; 6% of sulphur is oxidized in the gas phase and 44% in the aqueous phase. *a*, Fixed supersaturation $S_c = 0.3\%$, *b*, Variable supersaturation $S_c = 0.3 \pm 0.2\%$.

oxidation model^{19,20} shows that the rate of oxidation of SO_2 in cloud drops is dominated by H_2O_2 for $\text{pH} < 5.5$ and is therefore independent of acidity. In this case the oxidation rate is proportional to the drop volume. Inclusion of this volume dependence is expected to have a small effect on the number of new CCNs because, as shown in laboratory experiments, the increase in the dry particle size happens mainly in a single cloud cycle and is large enough to form an efficient CCN for any typical cloud drop size²¹. Models for oxidation of sulphur in clouds in the presence of heterogeneous chemistry of the dry aerosol particles show the ability of large non-acid cloud drops to scavenge part of the sulphur^{17,18}. The effect was shown to depend on the concentration and size of salt particle. For a distribution of salt particles that is typical of Cape Grim, Australia¹⁸, the simulated increase in the CCN concentration in one cloud process for 0.5% supersaturation was half ($\Delta\text{CCN}/\text{CCN} = 15\%$) that without the salt particles¹⁷ (30%). For a salt particle distribution typical of the Pacific²² and Atlantic¹³ Oceans, the increase in CCN was similar with and without the salt particles¹⁷. Therefore the uncertainty in the effect of aerosol heterogeneous chemistry may decrease the available sulphur for in-cloud generation of new CCNs by 0–50%, and reduce the concentration of new CCNs accordingly.

Coagulation between particles and in-cloud scavenging was introduced to the model using size-dependent rates developed by Hoppel¹³. These account for the particle brownian diffusion and gravitational collection of interstitial particles by cloud droplets.

To test the model we simulated measured aerosol size distributions^{13,14}. Figure 1a shows the time evolution of the size distribution for maritime conditions, that is low initial particle concentration, supersaturation based on a boxcar probability distribution and low SO_2 concentration ($0.5 \mu\text{g-S m}^{-3}$). In Fig. 1b the final size distribution is shown for a variety of conditions. It shows the effect of replacing the boxcar distribution of S_c with a gaussian distribution, and the effect of adding a nuclei mode to the initial size distribution. The size distribution is compared with measurements of maritime air¹³ (Figs 7 and 10 in ref. 13). We are able to reproduce the measured two aerosol size modes found in the measurements^{13,14}, at $0.015\text{--}0.03 \mu\text{m}$ for particles generated in the gas phase, and at $0.08\text{--}0.12 \mu\text{m}$ for particles that grew in the aqueous phase in clouds (Fig. 1b). For a polluted environment, that is high initial particle concentration and larger SO_2 concentration ($1 \mu\text{g-S m}^{-3}$), a main mode that combines

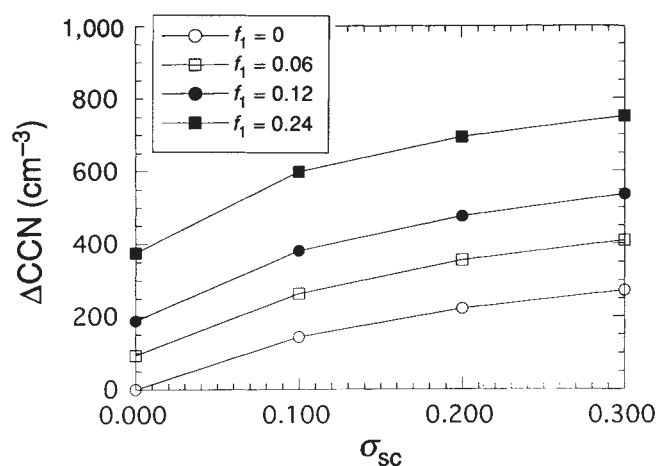


FIG. 3 Concentration of new CCN, ΔCCN after 10 cloud cycles due to anthropogenic activity as a function of the standard deviation of supersaturation σ_{sc} and the fraction of SO_2 oxidized into new particles, f_1 . The initial SO_2 concentration is $1 \mu\text{g-S m}^{-3}$, the initial number of particles is 800 cm^{-3} and the average supersaturation is 0.3%.

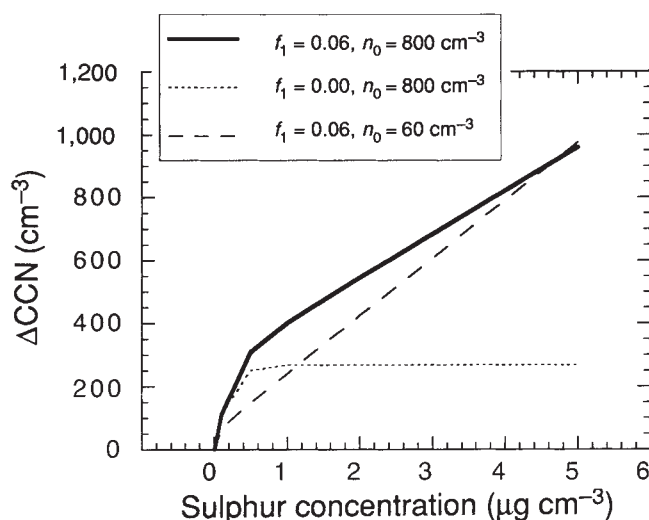


FIG. 4 The concentration of new CCN, ΔCCN , as a function of the initial sulphur concentration for a variable supersaturation with a gaussian probability distribution $0.3 \pm 0.2\%$, the fraction of sulphur oxidized in gas-phase, f_1 , and the initial number of particles in the accumulation mode, n_0 , is given for each plot.

these two processes appears at $0.06\text{--}0.08 \mu\text{m}$, also in agreement with measurements^{13,14} (Fig. 2).

The effect of varying the supersaturation ($S_c = 0.3\% \pm \sigma_{sc}$) on the new CCNs generated by the anthropogenic activity, ΔCCN , are shown in Fig. 3, for four values of f_1 . ΔCCN depends linearly on f_1 , but also depends significantly on σ_{sc} . For example, the efficiency of anthropogenic SO_2 in generating new CCNs with $f_1 = 0.06$ is four times larger for $\sigma_{sc} = 0.2$ than for $\sigma_{sc} = 0.0$. Therefore, anthropogenic sulphur with $f_1 = 0.24$ and no variations in the supersaturation ($\sigma_{sc} = 0$) generates as many new CCNs as anthropogenic sulphur for $f_1 = 0.06$ but with variations in the supersaturation of $\sigma_{sc} = 0.2$. The results shown in Fig. 4 test the effect of σ_{sc} on the dependence of ΔCCN on the sulphur level. For $f_1 = 0$ the effect of anthropogenic sulphur on ΔCCN saturates for $M_s = 0.5 \mu\text{g-S m}^{-3}$ because there is a limited availability of small particles to be activated. For $f_1 = 0.06$, ΔCCN increases steadily with an increase in M_s for $\sigma_{sc} = 0.2$, although at a slower rate than for low values of M_s . Whereas for $M_s = 1 \mu\text{g-S m}^{-3}$, variations in the supersaturation for $\sigma_{sc} = 0.2$ quadrupled the concentration of new CCN, for $M_s = 5 \mu\text{g-S m}^{-3}$ the concentration only doubles that for $\sigma_{sc} = 0$.

Climate models that describe the effects of aerosols on climate need to address many physical and chemical processes, and therefore are forced to simplify. But 'secondary' processes such as the variability in the supersaturation may have a dominant effect on the results of the climate calculations. One possible mechanism for the variations in supersaturation experienced by a particle is cycling through continental cumulus clouds before serving as a CCN in a maritime stratus cloud¹⁰. Indirect measurements indicate supersaturations of 0.5–5% in cumulus clouds²³ and $<0.5\%$ in stratus clouds¹⁰. Our calculations show that even small variations in the supersaturation ($0.3 \pm 0.2\%$), that are expected to occur in maritime stratus clouds, are sufficient for the in-cloud oxidation to quadruple the effect of anthropogenic emission of SO_2 on climate through cloud processes. Therefore, this effect of sulphate on climate may be similar to the levels predicted previously^{2,9,24}. There are many uncertainties in this result because of the complexity of heterogeneous sulphate chemistry and the possible competition of sulphates with other aerosol types, for example organic aerosol^{25,26}. But it is our opinion that before the 'details' of the model pre-

dictions (for example, variations in the supersaturation) are examined closely, it is too early to dismiss the importance of the effect of sulphate–cloud interaction on climate. It is possible that the models will not be able to assess this effect before enough measurements on the relationship between chemistry, aerosol size distribution and CCNs are obtained. □

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- Twomey, S. A., Piepgrass, M. & Wolfe, T. L. *Tellus* **36b**, 356–366 (1984).
- Kaufman, Y. J., Fraser, R. S. & Mahoney, R. J. *Clim.* **4**, 578–588 (1991).
- Charlson, R. J. *et al.* *Science* **255**, 423–430 (1992).
- Leaith, W. R., Isaac, G. A., Strapp, J. W., Banic, C. M. & Wiebe, H. A. *J. geophys. Res.* **97**, 2463–2474 (1992).
- Hansen, J. E. & Lacis, A. A. *Nature* **346**, 713–719 (1990).
- Twomey, S. A. *Atmos. Envir.* **25A**, 2435–2442 (1991).
- Langner, J., Rodhe, H., Crutzen, P. J. & Zimmermann, P. *Nature* **359**, 712–715 (1992).
- Kiehl, J. T. & Briegleb, B. P. *Science* **260**, 311–314 (1993).
- Wigley, T. M. L. *Nature* **339**, 355–357 (1989).
- Hegg, D. A. *Geophys. Res. Lett.* **17**, 2165–2168 (1990).

- Twomey, S. A., *Atmospheric Aerosol* (Elsevier, Amsterdam, 1977).
- Hobbs, P. V. *Q. Jl. R. met. Soc.* **97**, 263–271 (1971).
- Hoppel, W. A., Fitzgerald, J. W., Ferick, G. M. & Larson, R. E. *J. geophys. Res.* **95**, 3659–3686 (1990).
- Hegg, D. A., Ferek, R. J. & Hobbs, P. V. *J. geophys. Res.* **98**, 8841–8846 (1993).
- Whitby, K. T. *Atmos. Envir.* **12**, 135–159 (1978).
- Lelieveld, J. & Heintzenberg, J. *Science* **258**, 117–120 (1992).
- Hegg, D. A., Yuen, P.-F. & Larson, T. V. *J. geophys. Res.* **97**, 12927–12933 (1992).
- Ayers, G. P. & Larson, T. V. *J. atmos. Chem.* **11**, 143–167 (1990).
- Schwartz, S. E. *Atmos. Envir.* **22**, 2491–2499 (1988).
- Schwartz, S. E. in *The Chemistry of Acid Rain: Sources and Atmospheric Processes* (eds Johnson, R. W. & Gordon, G. E.) Ch. 8, 94–108 (ACS Symp. Ser. No. 349, Atmospheric Chemistry Soc., 1987).
- Hoppel, W. A., Frick, G. M. & Fitzgerald, J. W. *Aerosol Sci. Tech.* **20**, 1–30 (1994).
- Clarke, A. D., Ahlquist, N. C. & Covert, D. S. *J. geophys. Res.* **92**, 4179–4190 (1987).
- Paluch, I. R. & Knight, C. A. *J. Atmos. Sci.* **41**, 1801–1815 (1983).
- Kaufman, Y. J. & Chou, M.-D. *J. Clim.* **6**, 1241–1252 (1993).
- Novakov, T. & Penner, J. E. *Nature* **365**, 823–826 (1994).
- Hegg, D. A., Hobbs, P. V., Ferek, R. J. & Waggner, A. P. *J. geophys. Res.* (Submitted).

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Rising temperatures in the subtropical North Atlantic Ocean over the past 35 years

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As an oceanographic contribution to the quinquennial celebrations of Columbus's voyage of discovery in 1492, a collaborative expedition was carried out in July–August 1992 to make a transatlantic, deep-ocean hydrographic section along Columbus's route at 24° N. The 24° N section is of interest for studies of climate change because it has been surveyed twice before, during the International Geophysical Year of 1957¹ and during 1981², and hence represents one of the best known of all oceanographic sections. Here we use the temperatures from all three surveys to show that the waters between 800 and 2,500 m depth have consistently warmed over the past 35 years and that the warming since 1957 is remarkably uniform across the east–west extent of the North Atlantic. The maximum warming, found at 1,100 m depth, is occurring at a rate of 1 °C per century. This trend is broadly consistent with model predictions of climate change due to increases in atmospheric CO₂ concentration^{3,4}, but the observed warming occurs in the interior ocean, in contrast to the surface-warming predicted by the models. The observed patterns of decadal-scale changes in ocean temperature are thus powerful signatures that can help us to understand the nature and causes of climate change.

To carry out the transatlantic hydrographic section, the Spanish naval vessel *Hespérides* departed Cádiz on 13 July 1992, proceeded to the Canary Islands taking three test stations en route, and after a brief stop in Las Palmas began the first station at the 50 m isobath off the coast of Africa at 24° 30' N on 20 July. Proceeding westward along 24° 30' N, we stopped for 101 hydrographic stations at ~33 nautical mile intervals up to the North American continental slope, 2 miles offshore of the Bahamas, to complete the mid-ocean section. Finally, 11 hydro-

graphic stations across Florida Straits were taken to survey the Gulf Stream flow at 26° N before *Hespérides* docked in Miami on 16 August. Each hydrographic station consisted of a full ocean depth conductivity–temperature–depth (CTD) station providing continuous measurements of pressure, temperature, salinity and oxygen throughout the water column, and of 24 water samples distributed through the ocean depth that were analysed on board for oxygen and salinity (to calibrate the CTD profiles), nitrite, nitrate, phosphate, silica, chlorofluorocarbons and trace metals. Here we concentrate on analysis of the new temperature measurements on the mid-ocean section between Africa and the Bahamas, and their differences from previous surveys in 1957 and 1981.

The three 24° N transatlantic sections differ in details of the sampling, primarily in station spacing: the 1957 section consisted of 38 Nansen bottle stations with typically 25 sampling depths on each station and an average station separation of 160 km (ref. 1); the 1981 section of 90 vertically continuous CTD stations for an average station separation of 70 km (ref. 2); and the 1992 section of 101 continuous CTD stations for an average separation of 60 km. One additional sampling difference is that the 1981 section angled southwestward from the Moroccan coast at 28° N to join 24° 30' N at 24° 18' W due to diplomatic clearance problems, whereas the 1957 and 1992 sections followed 24° 30' N essentially all the way across the Atlantic. To examine the differences between the surveys, each of the sections is interpolated to 35 levels in the vertical, and zonally to a 0.5° longitude grid; temperature differences between each of the sections are smoothed horizontally to eliminate eddy variability. Contoured sections of the smoothed temperature difference are presented: only from 24.5° W to 75.5° W for the 1992–81 and 1981–57 differences (due to the northward departure of the 1981 section east of 24.5° W), but from 16.5° W to 75.5° W over the complete 24° N transatlantic section for the 1992–57 difference (Fig. 1a–c).

Comparison of the 1981 and 1957 surveys had revealed that the ocean waters along 24° N had warmed appreciably down to 3,000 m depth over that 24-yr period⁵. Our version of the 1981–57 temperature difference (Fig. 1a) shows essentially the same feature as that earlier analysis because it is the same data and nearly the same calculation except that the earlier analysis used objective mapping for horizontal interpolation and a somewhat wider gaussian filter that did not diminish the temperature difference near the boundaries. We show the 1981–57 difference section not only for completeness but also to point out that the dominant feature in the temperature difference is the large region of strong warming extending down to 4,000 m depth in the central western basin, with temperature increases as large as 1 °C in the main thermocline.

From 1981 to 1992, the warming along 24° N in the Atlantic has continued (Fig. 1b). Notably, the entire eastern basin warmed appreciably over the past 11 years. In the western basin, the region of strong warming from 1957 to 1981 has now cooled (from 1981 to 1992); just to the west of this cooling is a region of approximately equal (but slightly greater) warming. Taking another look at the 1981–57 temperature difference (Fig. 1a), we note that just to the west of the strong warming there is indeed a cooling region in the earlier time period. It appears that there is a new type of large-scale, long-term variability evident in these temperature differences with zonally alternating regions of warming and cooling in the western basin of the Atlantic. The zonal scale of this variability from the longitude of maximum warming to longitude of maximum cooling is $\sim 1,000$ km. Although we have little idea of the timescale of such large-scale variability from the three 24° N sections, recent analysis of the long time series hydrographic station near Bermuda at 32° N,

65° W indicates a low-frequency, 10-yr periodicity in temperatures between 1,000 and 2,500 m depths (with minimum temperature in 1980) superimposed on a long-term warming trend⁶. Although we cannot precisely 'map' the Bermuda time series onto an exact longitude of the 24° N section, the variability appears to be of similar amplitude and in a similar depth range at both locations, so that we infer that the 1,000-km zonal scale variability in temperature changes observed in the western half of the 24° N section has a 10-yr timescale. Thus, there is a new form of large-spatial-scale, long-period variability found in the western basin of the North Atlantic that can complicate the measurement of climate change in the subtropical ocean.

Over the 35-yr period from 1957 to 1992, the 24° N section has warmed between 700 m and 2,500 m depths (Fig. 1c). What is particularly striking in the 35-yr warming is the flatness of the temperature-difference contours, that is the entire zonal extent of the 24° N section has warmed nearly uniformly, with the

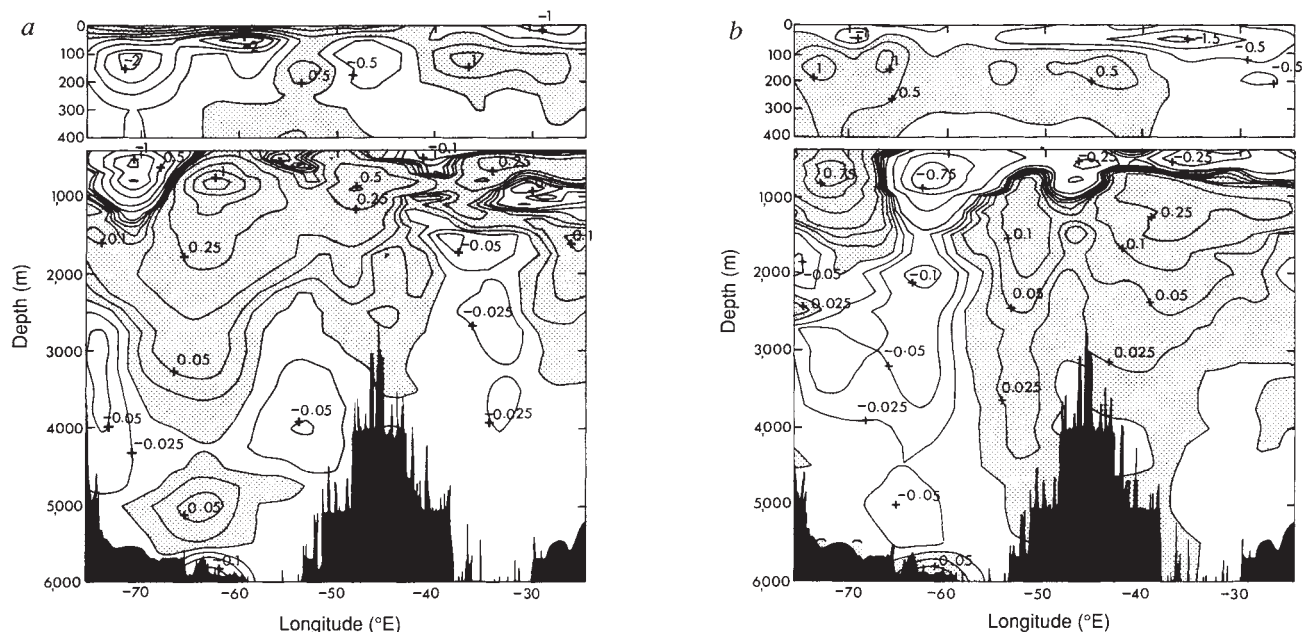
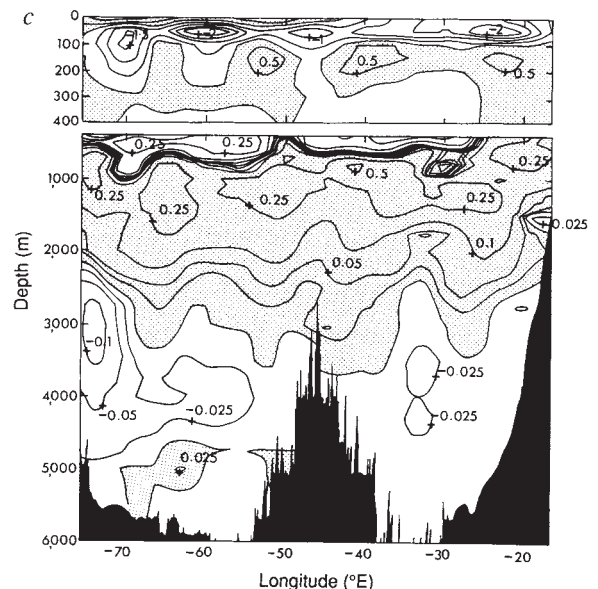


FIG. 1 Decadal-scale temperature changes (°C) in the subtropical North Atlantic ocean at 24° N: a, temperature difference 1981–57; b, temperature difference 1992–81; c, temperature difference 1992–57. Shading indicates warming over time.

METHODS. For each of the surveys in 1957, 1981 and 1992, temperature values are interpolated onto 35 standard levels from 0 to 6,000 m depth and onto a horizontal grid at 0.5° longitude intervals. The standard level values of temperature and salinity at each station for the 1957 survey are interpolated vertically from the discrete water sample data. The standard level values at each station for the 1981 and 1992 surveys are selected from the continuous conductivity–temperature–depth (CTD) profiles. Then, horizontally, cubic spline interpolation of the station data at each standard level is carried out to map each survey onto the 0.5° longitude grid. Differences in temperature between surveys of the gridded values are taken with care to ensure that temperature differences are considered only for those grid points where both surveys have interpolated temperature values. Horizontal smoothing of the temperature differences is then carried out by applying a gaussian filter of e-folding scale 300 km to eliminate mesoscale eddy variability. Based on the argument that the null value of temperature difference is zero, we apply the gaussian filter over the complete zonal extent of the 24° N section with zeros assumed at the edges of the section and in the Mid-Atlantic Ridge, where there are no values of temperature difference. The result is a diminished size to the temperature near boundaries due to averaging-in more and more zeros during the gaussian filtering. Each figure is split into an upper panel, where short-term, seasonal changes may cause the temperature differences in the upper 400 m, and a lower panel where long-term, decadal changes cause the temperature differences in the intermediate and deep waters.



maximum warming (up to 0.5°C) occurring at a depth of $\sim 1,000$ m. The deeper water below 3,000 m depth has cooled, with the strongest cooling of 0.1°C appearing just below 3,000 m depth at the western boundary where the southward flow of North Atlantic Deep Water (from its formation in the Greenland and Labrador Seas) is renewing the deep water reservoir⁷.

Vertical profiles of the zonally averaged warming for the three periods (Fig. 2) indicate both warming and cooling in the upper few hundred metres of the water column, which may be due to the different sampling times within the annual cycle of the three sections. For all three time periods there is consistent warming between 800 and 2,500 m depths. The maximum warming occurs at 1,100 m depth and reaches as high as 0.32°C over 35 yr, or $\sim 0.009^{\circ}\text{C yr}^{-1}$. Below 3,000 m depth, there is consistent cooling of the deep waters for all three time periods but the maximum cooling is only 0.03°C over 35 yr at 3,750 m depth, an order of magnitude less than the warming at 1,100 m.

On the basis of the error analysis described in Fig. 2, the warming from 1957 to 1992 is statistically significant between 800 m and 2,250 m depth. Over this depth range, the vertically integrated heat content has increased by $1 \times 10^9 \text{ J m}^{-2}$, or at an average rate of 1 W m^{-2} over the 35 yr between the 1957 and 1992 surveys. The change in dynamic height over this depth interval, which is the effective sea level rise due to the warming, is 3 dynamic centimetres. Such calculations illustrate how even

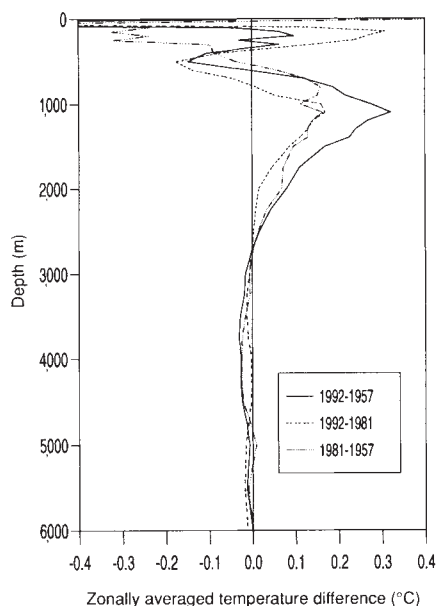


FIG. 2 Vertical profiles of the zonally averaged temperature differences ($^{\circ}\text{C}$) over the time periods 1981-57, 1992-81 and 1992-57 in the subtropical North Atlantic ocean at 24°N . At each level, the average and standard deviation of the gridded temperature differences before filtering are calculated from 24.5°W to 75.5°W for the 1992-81 and 1981-57 differences, and from 16.5°W to 75.5°W for the 1992-57 differences. The standard deviations of the temperature differences, which decrease from 0.44°C at 800 m depth, to 0.07°C at 3,000 m depth, to 0.035°C at 5,000 m depth for the 1992-57 period, are due principally to mesoscale eddy fluctuations which have a horizontal length scale of ~ 100 km (ref. 8). The standard error uncertainty in the zonally averaged temperature difference can then be estimated by dividing the standard deviation by the square root of the number of mesoscale eddy length scales in the 6,000-km width of the 24°N section. Using a criterion that the zonally averaged temperature difference is statistically significant when it exceeds three standard errors, we conclude that the observed warming from 1957 to 1992 is significant between 800 and 2,250 m depth, and that the cooling is significant between 3,500 and 4,500 m depth. More sophisticated objective mapping techniques yield essentially similar conclusions⁸.

a very small warming (when compared to local air-sea heat exchanges) can cause substantial changes in sea level when it continues over a long time period.

There have also been changes in salinity; the changes at each depth are principally such as to maintain the temperature-salinity relationships of the various water masses⁸. Thus, to first order, the warming at each depth from 1957 to 1992 is due to a deepening of the isotherms (and isohalines and isopycnals) by 50-70 m in the depth range 1,000-1,500 m. The extent to which the temperature-salinity and temperature-oxygen relationships for the various water masses are changing is an important issue in ocean climate change⁹ and we believe there are measurable changes in these relationships⁸. The issue, however, is a subtle one requiring complex analysis techniques¹⁰ as well as careful assessment of changes in measurement techniques and standards over time¹¹.

Lastly, because of the zonal uniformity of the temperature changes along the 24°N section, we find little change over time in the geostrophic, mid-ocean meridional circulation across 24°N . The vertical-meridional overturning circulation across 24°N in the Atlantic, which carries $\sim 1.2 \times 10^{15} \text{ W}$ of heat poleward^{2,12}, is a major component in the global 'conveyor belt'¹³. Because of the similarity in the mid-ocean geostrophic circulations estimated from the three surveys⁸, we do not expect that this heat transport has changed substantially since 1957.

These observations of rising temperature have profound implications for modelling climate change. Most climate models that include the deep ocean seek first to achieve an equilibrium ocean circulation under present wind and buoyancy forcing conditions. Some models maintain deep ocean temperature and salinity characteristics, as determined for example in the Levitus climatology¹⁴ which in the subtropical North Atlantic is heavily based on the International Geophysical Year (IGY) 1957 survey of which the first 24°N section was part. These observations of substantial ocean temperature change suggest first that the deep ocean is not in an equilibrium state and that it has changed significantly under the wind and buoyancy forcing conditions of the past 35 yr.

Second, the observed warming from 1957 to 1992 at 24°N is comparable to the rate at which ocean sea surface temperatures are expected to change due to CO_2 doubling on the basis of climate models. Commonly, sea surface temperatures are found in the models to increase by $\sim 2.5^{\circ}\text{C}$ over about a century of CO_2 doubling¹⁵⁻¹⁷. Here we find the zonally averaged ocean temperature at 1,100 m depth at 24°N in the Atlantic has increased by 0.32°C in 35 years, or at a rate of just about 1°C per century.

Last, the patterns of long-term temperature changes in the ocean can provide a powerful means for assessing models of climate change. Although the warming at 24°N found here might be quickly taken to confirm projections of global warming due to CO_2 increase, the observed warming is surprisingly deep in the interior ocean in comparison with the surface intensified warming of ocean climate models^{3,4}. A similar analysis of modern and historical hydrographic sections across the subpolar North Atlantic found cooling within the subpolar gyre¹⁸, which seemed initially at odds with global warming. Yet for some climate models, cooling in the subpolar gyre and warming in the subtropical gyre is the signature of the climate change due to CO_2 increase^{4,19}. A comprehensive resurvey of the North Atlantic ocean for comparison with earlier surveys would provide a benchmark for understanding the nature of climate change. The systematic IGY survey of the North Atlantic ocean in 1957-58^{1,20} is arguably the earliest oceanographic survey at modern-day measurement accuracy and the North Atlantic ocean with its deep and intermediate water formation is a prime focus for understanding the thermohaline circulation and its climate variability over decadal time scales²¹. A systematic hydrographic survey of the North Atlantic in 1997 would determine the magnitude and spatial scales of ocean climate change over the 40 yr since the IGY survey of 1957, a period during which reasonably

consistent estimates of the wind and buoyancy forcing should be available from the re-analysis activities now underway at international forecasting centres. It should also provide a useful testbed for development of coupled atmosphere-ocean models of climate change. □

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1. Fuglister, F. C. *Atlantic Ocean Atlas* (Woods Hole Oceanographic Instrn, Woods Hole, 1960).
2. Roemmich, D. & Wunsch, C. *Deep-Sea Res.* **32**, 619–664 (1985).
3. Manabe, S., Stouffer, R. J., Spelman, M. J. & Bryan, K. J. *Climate* **4**, 785–818 (1992).
4. Mikolajewicz, W., Santer, B. D. & Maier-Reimer, E. *Nature* **346**, 589–593 (1990).
5. Roemmich, D. & Wunsch, C. *Nature* **307**, 447–450 (1984).
6. Joyce, T. M. *Deep-Sea Res.* (in the press).
7. Fine, R. A. & Molinari, R. L. *Deep-Sea Res.* **35**, 1441–1450 (1988).
8. Lavin, A. M., Thesis, Massachusetts Inst. Technol. (1993).
9. Bindoff, N. L. & Church, J. A. *Nature* **357**, 59–62 (1992).
10. Bindoff, N. L. & McDougall, T. J. *J. Phys. Oceanogr.* (in the press).
11. Mantyla, A. W. *Deep-Sea Res.* (in the press).
12. Bryden, H. L. in *Interactions Between Global Climate Subsystems* (eds McBean, G. A. & Hantel, M.) 65–75 (Geophys. Monogr. No. 75, Am. Geophys. Un., Washington DC, 1993).
13. Broecker, W. S. *Oceanography* **4**, 79–89 (1991).
14. Levitus, S. *Climatological Atlas of the World Ocean* (NOAA Prof. Pap. No. 13, US Govt. Print. Off., Washington DC 1982).
15. Schlesinger, M. E. & Mitchell, J. F. B. *Rev. Geophys.* **25**, 760–798 (1987).
16. Bretherton, F. P., Bryan, K. & Woods, J. D. in *Climate Change The IPCC Assessment* (eds Houghton, J. T., Jenkins, G. J. & Ephraums, J. J.) 173–193 (Cambridge Univ. Press, 1990).
17. Manabe, S. & Stouffer, R. J. *Nature* **364**, 215–218 (1993).
18. Read, J. F. & Gould, W. J. *Nature* **360**, 55–57 (1992).
19. Manabe, S., Bryan, K. & Stouffer, R. J. *J. Phys. Oceanogr.* **20**, 722–749 (1990).
20. Dietrich, G. *Atlas of the Hydrography of the Northern Atlantic Ocean* (Conseil International Pour L'Exploration de la Mer, Charlottenlund Slot, Denmark, 1969).
21. Lehman, S. *Nature* **365**, 108–110 (1993).

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Discovery of a pure rhenium mineral at Kudriavy volcano

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KUDRIAVY volcano on Iturup island in the Kuril arc is an active calc-alkaline volcano. It has not erupted this century; its current volcanic activity is characterized by hot (up to 910 °C) gas jets which have been stable for at least 30 years. The composition of the gaseous emissions is typical of high-temperature fumaroles, but we report here the discovery of unusual subsurface sublimates associated with one gas jet—a sulphide mineral containing rhenium as the only cation. To our knowledge, this is the first reported occurrence of a pure rhenium mineral. The concentration of rhenium in the fumarole gas is only 2–10 p.p.b., so the condensation of pure rhenium sulphide from this gas requires both enrichment of rhenium by eight orders of magnitude and remarkable selectivity. Rhenium is generally believed to exist in only trace amounts at the Earth's surface, but our findings demonstrate that it can be readily mobilized, dispersed and concentrated by degassing magmas.

The andesito-basaltic Kudriavy volcano (986 m) is located on the northern tip of Iturup island in the Kuril volcanic arc, which divides the Okhotsk sea and Pacific ocean. The last recorded explosion took place in 1883. (A later date of 1949 attributed to the last eruption is probably not correct, as remains of an

early twentieth century Japanese sulphur mine can still be seen in the crater.)

The volcano was visited for a field trip in 1961, and since 1989 annual field work has been undertaken. We have made temperature measurements on the volcano since 1990. In that year the highest temperature measured was ~770 °C; in 1991 we managed to penetrate into the 'hot zone' which yielded a fumarole temperature of ~910 °C (Fig. 1). In 1992, this fumarole yielded the same temperature, and a new fumarole with a temperature of 940 °C was identified. This is probably the maximum stable temperature of fumaroles measured over a continuous period.

No noticeable changes of temperature or the power of gas emission have been detected since 1961 and during 1989–93. Repeated temperature measurements of individual fumaroles over the last 5 years gave the same results, within the error of measurement. As the temperature of the jets and the power of the gas emission appear to be constant, we conclude that the process of magmatic degassing is itself essentially constant.

Gas samples were taken using the Gigenbach method², trapping H₂S by cadmium acetate. Gas compositions³ of the high-temperature fumaroles are typical of the island arc volcanoes: 95 mol% H₂O, 3–4 mol% CO₂, ~1 mol% SO₂, 0.4–0.7 mol% H₂S, with H₂, HCl and HF present in smaller quantities. CO, CH₄ and so on are present in minimal amounts. Excluding H₂O and CO₂, a constant content of HCl and HF is found (20 ± 1% and 1 ± 0.1%, respectively). This constancy of the concentrations of salt hydrolysis products in a gas phase provides further evidence that the source area of the gas is approximately isothermal.

Oxygen fugacity measurements were made⁴ of gas emitted from a fumarole at 890 °C (Fig. 1) over a period of 4 h, using a cell with a solid electrolyte, and yielded log f_{O_2} = -12.4 ± 0.1. Values of f_{O_2} calculated from the ratios H₂S/H₂/SO₂ and CO/CO₂ in the gas phase from this and other fumaroles are in agreement with the direct measurements, and over the temperature range 500–950 °C approximate to log f_{O_2} = 0.0212T - 31.02, where T is the temperature in °C. Within this temperature interval the f_{O_2} of the gas jets are close to the Ni/NiO buffer. So, the main fluid components are water and carbon dioxide.

Samples of condensed vapour from hot gas jets were analysed by the inductive coupled plasma (ICP) approach⁵. The condensates were collected using a water-cooled glass vessel. The

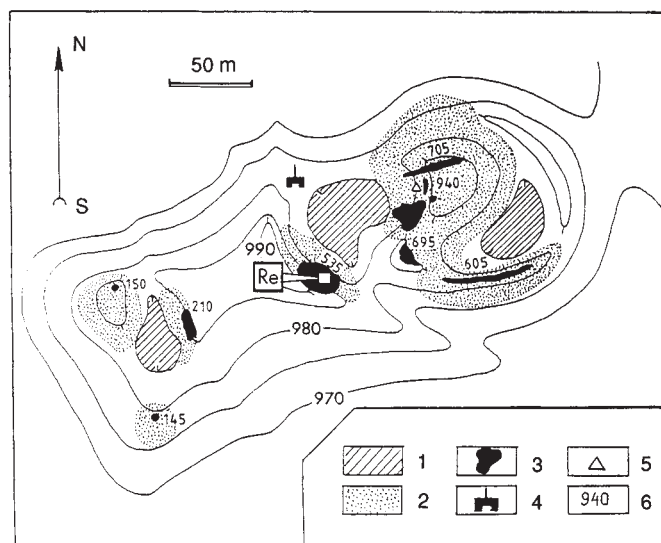


FIG. 1 Map of fumarole fields at the crater of Kudriavy volcano, Iturup island, Kuril arc (148° 45' E, 45° 41' N). Legend: 1, detrital deposits; 2, sulphur deposits; 3, fumarole fields; 4, remains of Japanese sulphur mine; 5, location of f_{O_2} measurements; 6, temperatures of fumaroles (°C) and altitude (on contours, m). Location of Re mineralization indicated by Re symbol and white square.

condensates are rich in sulphur, which both separates out as a precipitate, and is present in solution in the form of H_2SO_4 with a concentration in the range 200–4,000 p.p.m. Chlorine was not analysed by ICP, but appears to be mainly in the form of HCl. The concentrations of trace components are: Si, 80–200 p.p.m.; B, 20–40 p.p.m.; Na, 10–50 p.p.m.; K, 10–100 p.p.m.; and Ca, Fe, Al from 2 to 20 p.p.m.. Analyses of the pure aqueous solution phase of the condensate gave variable contents of ore metals; for example, the content of Ag ranges from 2 to 6 p.p.b.. However for the same sample, analyses of the aqueous solution together with the sulphur-rich sediment (shaken sample without any additions) yield metal concentrations that are some two orders of magnitude higher than in the aqueous phase alone. This is observed for Cu, As, Sb, Te, Tl, Pb and Bi. Most of these elements precipitate at sampling together with sulphur, possibly in the form of sulphides. The Re content in both the aqueous solution and the sulphur-rich precipitate is 2–8 p.p.b..

After complete condensation of fumarole gases and the subsequent evaporation of the water from the hot ground, a dry residue should remain containing up to 100 kg of ore metals per ton of residue (that is, up to 18 kg of Tl, ~1 kg of Ag + In + Pd, and several kilograms each of Sn, Bi, Te, Hg and Zn).

Before sampling the gas, we usually removed the rubble from around the jet to insert a marker pipe. When one fumarole with a temperature of 535 °C (Fig. 1) was prepared in this way for sampling, we found sublimates on the rubble at a depth of 25–40 cm. The mineral covers fragments of pyroclastic debris, lava and altered rocks with a solid crust 1–2 mm thick, and is light-grey, graphite-like and soft. The scanning electron micrographs of the deposits (Fig. 2) reveal a plate-like morphology typical of sublimates.

On the basis of its appearance, the mineral was diagnosed initially as molybdenite, MoS_2 , which is often found in volcanic

sublimates. In the field, we therefore did not see any need for a detailed investigation of the sequence of mineral precipitation. But during subsequent microprobe studies of the mineral, we found that it does not contain any Mo.

More than 50 analyses of different samples of this sublimate have now been made. Well pressed and polished samples yield 76–78 wt% Re and 23–24 wt% S, that is, between the theoretical compositions of ReS_2 (74.4% Re) and Re_2S_3 (79.5% Re). The basal spacing is 6.11 ± 0.01 Å. The mineral is currently being analysed in detail at the Institute of Ore Deposits and Mineralogy at the Russian Academy of Sciences. A number of samples representing compositions intermediate between molybdenite and rhenium sulphide were also found; samples containing 5–12% of Mo are quite common. Molybdenite with a significant Re content has also been reported⁶.

The samples of sublimates were also collected on glass tubes (25 mm diameter, 1 m long) placed in the marker pipes. These tubes, cooled by wind and rain, became incrustated with rhenium sulphide, molybdenite and intermediate compositions of solid solutions $\text{ReS}_x\text{--MoS}_2$ over a period of 2 months. A variety of other minerals were also deposited, including halite, sylvite and magnetite.

Previously, Re minerals have been observed only as inclusions in pyrrhotite, pentlandite⁷ and other sulphides^{8,9}. Moreover, they contain Cu, Mo or Pb in addition to Re, and this appears to have precipitated from solid solutions. To the best of our knowledge, the sublimates from Kudriav volcano are the first natural mineral with Re as the only cation and the first example of macroscopic Re mineralization.

It is of interest to note that in the condensate solution the amount of Re does not exceed 10 p.p.b.. Thus, the process of condensation of rhenium sulphide from the gas which also contains high concentrations of other metals, requires not only Re enrichment by almost eight orders of magnitude, but also a remarkable selectivity.

The discovery of rhenium mineralization in sublimates is a clue to the form of Re transport. It takes place in the gas phase as no liquid solutions exist under crater conditions. The industrial technology of rhenium production is based on extracting it from gases emitted during copper and molybdenum processing. We can see here how nature partly reproduces an industrial process. The Re oxides have remarkably high vapour pressures ($p = 1$ atm is reached at 1,400 °C for ReO_2 , at ~600 °C for ReO_3 and at ~350 °C for Re_2O_7 (ref. 10)), itself sufficient for mass transport. Unfortunately, we cannot determine for certain the exact forms in which Re and other elements are transported. The concentration of each of the halogens (Cl, F, I) is greater than of all the ore elements together and might therefore play a role in transporting the rhenium. Industrially, gas transport of metals often uses iodide, chloride or fluoride complexes. Any of all of these processes may be significant at Kudriav gas jets.

The scale of rhenium transport is hard to estimate, but to a first approximation, if the total steam emitted is ~10–100 tons per day, rhenium precipitation should be measured in grams per day. □

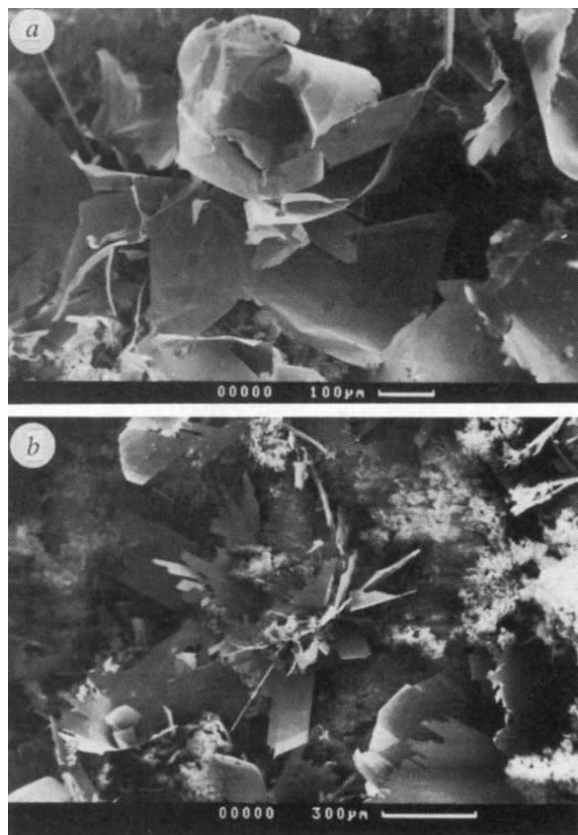


FIG. 2 Scanning electron micrographs of thin plates of rhenium sulphide. a, In the form of a rose, b, as radial clusters. (Scale bar is 100 µm in a, and 300 µm in b.)

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- Gorshkov, G. S. *Volcanism of Kuril Island Arc*, (Nauka, Moscow, 1967).
- Giggenbach, W. F. *Bull. volcan.* **39**, 132–145 (1975).
- Tkachenko, S. I. et al. *Dokl. Akad. Nauk SSSR* **325**, 823–828 (1992).
- Rosen, E., Osadchii, E. & Tkachenko, S. *Chem. Erde* **53**, 219–226 (1993).
- Korzhinsky, M. A., Tkachenko, S. I., Romanenko, I. A., Steinberg, G. S. & Shmulovich K. I. *Dokl. Akad. Nauk SSSR* **330**, 627–629 (1993).
- Bernard, A., Symonds, R. B. & Rose, W. I. *Appl. Geochem.* **5**, 317–326 (1990).
- Tarkian, M., Houslay, R.M., Volborth, A., Greis, O. & Moh, G. H. *Europ. J. Miner.* **3**, 977–982 (1991).
- Jedwab, J. & Fletcher, T. *Terra (Abstr.)* **3**, 107 (1991).
- Mitchell, R. H., Laflamme, J. H. G. & Cabri, I. J. *Miner. Mag.* **53**, 635–637 (1989).
- Bolshakov, K. J. (ed.) *The Technology of Rare and Trace Elements* Vol. 2 613–663 (Vishaya Shkola, Moscow, 1969).

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Constraints on North American plate velocity from the Yellowstone hotspot deformation field

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OCEANIC hotspot tracks generally indicate that the mantle plumes that give rise to them are fixed in position (relative to a lower-mantle reference frame) for timespans of ~ 100 Myr¹⁻³. It has not been clear whether continental hotspots are equally stable; for example, there is evidence that the velocity of the Yellowstone hotspot has been quite irregular with respect to the North American plate over the past 16 Myr⁴⁻⁷, but measurements of its velocity based on caldera locations have been too uncertain and irregular to be definitive. Here I describe a new method of obtaining the plume velocity, which uses the broader lithospheric effects of the hotspot rather than the locations of individual calderas. For the past 10 Myr, the results indicate a constant North American plate velocity of 2.2 cm yr^{-1} , which agrees with independent estimates from global plate motion inversions^{8,9}. This velocity is considerably lower than previous estimates obtained from caldera locations, and clearly differs from the much higher (but less well constrained) velocities of the previous 6 Myr (refs 5, 7).

The volcanic rocks of the Yellowstone plateau and eastern Snake River plain have long been recognized as the track of a continental hotspot^{1,4,10}. Estimates of the North American plate velocity based on the entire 16-Myr track of the hotspot range from 3.5 to 4.5 cm yr^{-1} (refs 4, 6, 11). A significant change in apparent velocity at 10 Myr has recently been recognized^{5,7}. Estimates of the velocity over the interval from 10 Myr to the present range from 2.9 to 3.43 cm yr^{-1} (refs 5, 7); between 16 and 10 Myr the 'apparent' rate (not accounting for subsequent extension) is estimated at 7 cm yr^{-1} (ref. 7). In addition, there is considerable debate as to the path of the Yellowstone hotspot before 10 Myr^{4,5,7,10,12,13}; Fig. 1 shows the three proposed tracks. After 10 Myr, however, the hotspot track is well constrained to the eastern Snake River plain and the Yellowstone plateau^{4,6,7,10,12,14,16}.

Most of these estimates of the North American plate velocities are based on the age and estimated locations of silicic volcanic rocks and their associated calderas. Unfortunately, these calderas do not progress sequentially. Moreover, the calderas formed between 10.3 and 4.3 Myr, the crucial interval for assessing plate velocity, are buried under 1–3 km of basalt. As a result, the Yellowstone hotspot track lacks the precision necessary for assessing plate velocity and, in part, constitutes the rationale for not including the Yellowstone hotspot in a fixed hotspot reference frame determination of plate velocity⁹.

Here I present an alternative method for assessing relative velocity, that uses the onset of accelerated fault movement initiated by the broader thermal interaction of the hotspot plume and the lithosphere. The method involves identifying precipitous changes in the displacement rate of normal faults adjacent to the track of the hotspot. The distance from each fault to the present position of hotspot-related deformation is plotted against the time interval of accelerated faulting. The intervals provide the constraints on lines whose slope represents the velocity. Care must be taken to distinguish between the onset of faulting and the onset of accelerated faulting, because many of the normal faults were active before the advent of the hotspot^{15,7}, whereas other faults were initiated by the thermal influences of

the hotspot^{7,14,16}. Anders *et al.*¹⁵ showed that the passage of the hotspot influences displacement rates on a fault $>75 \text{ km}$ from the hotspot track, and demonstrated an order-of-magnitude increase in displacement rates as the hotspot approached, followed by a decrease of two orders of magnitude after its passage. The technique used to assess relative displacement rate is fairly simple. The tectonic tilts of units of various ages in the footwalls and hanging walls are determined. The onset of high faulting rates is marked by sharp changes in the tectonic tilt of the various units sampled at a given site. For example, at one location a 6.6-Myr unit and a 4.3-Myr unit are conformable, and dip $\sim 30^\circ$ toward the fault. In the same section, a 4.0-Myr basalt dips 15° and two younger units (2.1-Myr and 1.5-Myr) are roughly conformable and dip $<2^\circ$ toward the fault. It is obvious that between 4.3 and 4.0 Myr there was a marked increase in faulting rate on this normal fault, the Grand Valley fault (Figs 2 and 3, site 5).

Using the technique outlined above, changes in displacement rates were assessed on seven large ($>1\text{--}3 \text{ km}$ displacement) normal faults to define the arrival of the faulting associated with the Yellowstone plume. These faults are the Beaverhead and Lemhi of east-central Idaho, the Grand Valley and Teton faults of eastern Idaho and western Wyoming, and the Madison, Hebgen and Centennial faults of southwestern Montana (Fig. 2). In this study, sites were chosen where units directly overlie one another, thereby eliminating the effects of differing fault geometry in defining the interval of accelerated faulting. Precipitous changes in relative tilt, not absolute tilt, can then be compared from site to site to define the onset of hotspot-related extension.

The intervals during which faults moved at accelerated rates are plotted in Fig. 3 as solid vertical lines. The length of these

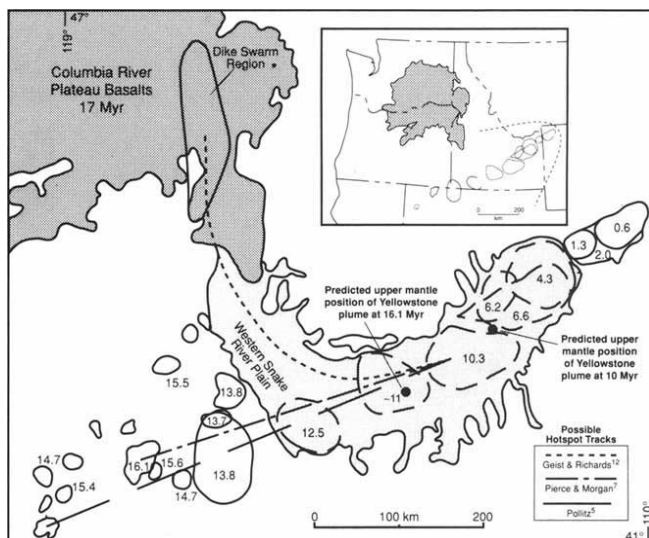


FIG. 1 Map of the northwestern United States showing the location of calderas used to define the track of the Yellowstone hotspot. The various possible tracks of the hotspot are shown as dashed lines and are discussed in the text. Also shown are the flood basalts of the Columbia River plateau (dark shading) and the region of the Snake River plain (light stippling). Circles represent the location of calderas, with dashed circles corresponding to calderas that are buried under 1–3 km of basalt. Solid circles are the expected locations of the Yellowstone plume at 10 and 16.1 Myr, assuming that this plume is fixed relative to the mantle and that there has been a constant 2.2 cm yr^{-1} North American plate velocity during the past 16 Myr (see text for further discussion). Area labelled 'Dike Swarm Region' is a likely source of the Columbia River flood basalts. Caldera locations are from Pierce and Morgan⁷. Dashed parabola on inset map is the outer parabola of Fig. 2.

vertical lines represents the restrictions on the onset of accelerated displacement rates. This length is more a function of the availability of rocks of the appropriate age with which to record changes in displacement rate than an error in measurement. Distances are measured on lines parallel to the propagation direction (N55° E) between the sampling site (solid circles in Fig. 2) and the intercept of the outer limit of hotspot-induced seismicity and latest Quaternary faulting from Anders *et al.*¹⁵. Differences in the exact location of the outer limit are not significant so long as the pattern of hotspot activity is symmetrical about the track of the hotspot, which is generally agreed on^{7,11,14,15}. Error in relative distance is reduced by using sampling sites as close to the centre of the hotspot track as possible. The two slopes in Fig. 3 represent the maximum and minimum value that can be accounted for by all the individual site data. The extreme slopes that fit all the data correspond to a velocity between 2.37 and 2.02 cm yr⁻¹, respectively, with an average value of 2.19 or 2.2±0.2 cm yr⁻¹. The percentage of post-10-Myr extension from faulting, calculated parallel to the hotspot track, is 16.2% near the hotspot's position at 10 Myr (ref. 17) and 0% extension at its most northeastern extent^{15,7}. Assuming

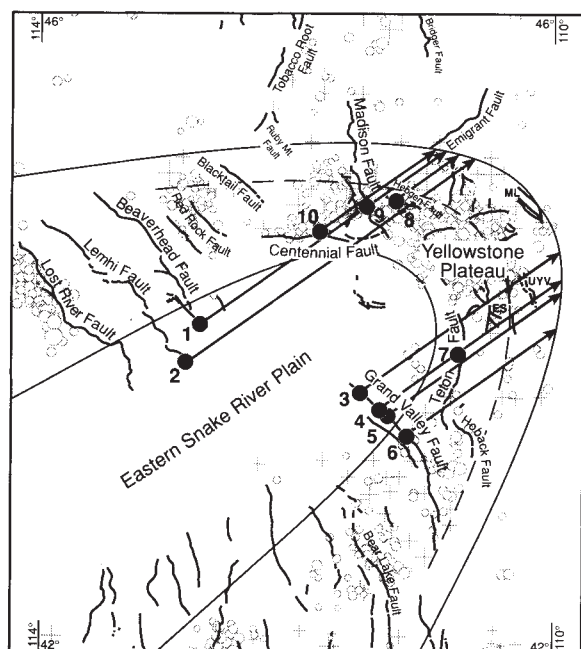


FIG. 2 Map of late Cenozoic normal faults and historic earthquakes of the region surrounding the eastern Snake River plain (see Fig. 1 for location map). Numbers 1 to 10 (solid circles) are the sampling sites. The two parabolas bound areas of increased seismicity¹⁵. The parabolic shape is probably due to the flow field created by the interaction of the moving lithosphere and the rising Yellowstone plume¹⁶. Dashed parabola is the estimated position of initiation of accelerated faulting based on the zero intercept of maximum velocity slope from Fig. 3. Arrows are parallel to the migration direction of the hotspot (N55° E) and represent the distance between sampling sites and the outer parabola. There can be significant variability in the exact location of the outer parabola because it is only necessary to establish the relative timing of the onset of hotspot related seismicity in order to assess velocity. However, the migration direction must be known, as it is, to within 2–3 degrees^{5,7,15} and because samples are compared from both north and south of the eastern Snake River plain, the pattern of seismicity must be symmetrical about the track of the hotspot (as generally agreed^{7,11,15}). Fault names are on the footwall blocks of respective faults. (Diagram modified from Anders *et al.*¹⁵ and Simpson and Anders²⁵.)

that the change in hotspot-parallel extension is also roughly linear, decreasing the hotspot-traverse distance by one half of the maximum extension yields a relative velocity for the North American plate of 2.0 ± 0.2 cm yr⁻¹. This value assumes an average slope between the maximum and minimum. However, Pierce and Morgan⁷ estimate that the start of the period of rapid extension on Teton fault was between 4.3 and 2.1 Myr. Recently collected data suggest an age slightly younger than 2.1 Myr, which is in accord with the maximum slope of 2.37 cm yr⁻¹. Correcting this value for extension yields a velocity of 2.17 or 2.2 ± 0.2 cm yr⁻¹, which, assuming the same error, is the same as the uncorrected average value.

A 2.2 ± 0.2 cm yr⁻¹ velocity on an azimuth of N55° E for the North American plate relative to the Yellowstone hotspot is a much closer agreement to the 2.2 ± 0.8 cm yr⁻¹ at N56° E determined using the global plate motion inversion model SH2 NUVEL1 (refs 9, 7) than any of the other estimates of velocity based on the assumed location of calderas, although Pierce and Morgan's⁷ caldera-based post-10-Myr velocity of

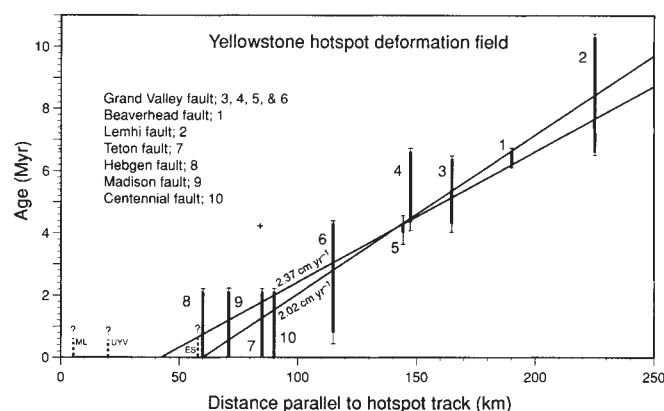


FIG. 3 Plot showing the progression of the Yellowstone deformation field. Vertical bars represent the interval when the onset of accelerated fault movement occurred. It is important to note that these vertical lines are not error bars, but rather are constraints on the timing of the onset of accelerated faulting. The most significant data are the most narrowly defined intervals, and these data exert the most control on the estimated rate of migration. Error bars are shown only for upper and lower bounds of the age intervals and represent the associated 2σ error. The two lines (2.37 and 2.02 cm yr⁻¹) represent the minimum and maximum velocity that fit all the data. Because the Madison, Hebgen, Centennial and Teton faults all exhibit high latest Quaternary displacement rates, it is necessary to define only the time of the onset of movement to provide an upper bound to the interval of accelerated faulting, which currently can only be limited to after 2.1 Myr (ref. 26). It is worth noting that there is an interlocking system of small-displacement (<1 km) faults surrounding the 0.6-Myr Yellowstone and 2.1-Myr Huckleberry Ridge calderas (only some of which are shown in Fig. 2). These faults either are directly traceable into the caldera (for example, faults labelled ES or UYV in fig. 2) or are concentric about the caldera (for example, faults labelled ML in fig. 2). These smaller faults are probably related to caldera rise and fall, which is significant enough to be measurable on the order of 50 yr (ref. 27) and therefore are not included with the data from large range-bounding normal faults. But if the onset of accelerated faulting preceded 2.1 Myr on the Teton faults (as suggested by Pierce and Morgan⁷; "+" indicates their upper bound) and if the smaller faults within 25 km of the Yellowstone caldera are symptomatic of broad-scale lithospheric extension rather than caldera collapse, then (K. L. Pierce, personal communication) there could be a change in the North American plate velocity at 4 Myr from 2.2 to 2.7 cm yr⁻¹ (corrected for extension). A higher plate velocity after 4 Myr is an intriguing possibility, but the constraints provided by the Madison, Hebgen and Centennial faults suggest that such a high velocity is unlikely.

$2.9 \pm 0.8 \text{ cm yr}^{-1}$ has error estimates that include the lower velocity. The SH2 NUVEL1 does not use the Yellowstone hotspot velocity, thus avoiding circularity in estimating rate, but does include an estimated direction.

The exact location of the Yellowstone plume under the North American plate is not known. Currently, geophysical techniques lack sufficient resolution to define its location¹⁸. The most recent eruption, the 0.6-Myr Yellowstone caldera, represents only the surface expression of the plume-generated thermal anomaly. Assuming that the Yellowstone caldera is directly over the plume's position at the time of eruption, the present upper-mantle position of the plume is estimated to be somewhere between 10 and 20 km to the northeast of the centre of the Yellowstone caldera. More likely, however, is that there is a time lag between eruption and the sub-lithospheric thermal perturbation caused by the plume¹⁶. The expected position of the plume can be estimated using the Yellowstone caldera and plate velocity determined by the intervals of accelerated faulting. In fact, all that is known about the position of the plume using these assumptions is that it is northeast of the calculated position by a distance that is a function of the lag time. Nonetheless, back-tracking the plume using this technique highlights why some workers have suggested a non-hotspot origin for the Yellowstone-Snake River plain volcanic province^{19,20}. As can be seen in Fig. 1, the best estimate for the location of the hotspot at 16.1 Myr based on a 2.2 cm yr^{-1} velocity is $>325 \text{ km}$ from its assumed position at the end of the track of rhyolitic volcanism^{7,21}. It has been suggested that this discrepancy can be accounted for by significant regional extension between 16.1 and 10 Myr (ref. 6), by deflection of the plume head by traction with the subducted Farallon plate¹², or by deflection of a buoyant plume head up-dip along the underside of the Farallon plate (K. Pierce, personal communication). Recently, it has been suggested that flood basalts are the result of a rising plume head, and that the associated tracks of volcanism represent the plume tail or deep mantle conduit²²⁻²⁴. However, models invoking deflection of the plume head to beneath the 17-Myr Columbia River flood basalts¹² do not have an associated track of rhyolitic volcanism linking them to the post-10-Myr hotspot track (Fig. 1); models that call for the hotspot head impinging under the McDermitt caldera do not successfully explain why the flood basalts are older than, and located 400 km to the northeast of, the McDermitt caldera^{7,21}.

The difficulties in applying a model of linear progression of hotspot volcanism²²⁻²⁴ to the Yellowstone hotspot between 17 and 10 Myr probably reflect a complex interaction between the rising mantle plume and the lithosphere. But from 10 Myr to the present, the velocity of the deformation field is constant, and matches the best estimates of the North American plate velocity based on global plate motion inversion studies^{8,9}. This suggests that the Yellowstone plume was a fixed sub-lithospheric source during this interval. □

16. Anders, M. H. & Sleep, N. H. *J. geophys. Res.* **9**, 15379–15393 (1992).
17. Anders, M. H., Spiegelman, M., Rodgers, D. W. & Hagstrum, J. T. *Tectonics* **12**, 1451–1459 (1993).
18. Dueker, K. Humphreys, E. *Geophys. Res. Lett.* **17**, 1327–1330 (1990).
19. Hamilton, W. & Myers, W. B. *Rev. Geophys.* **4**, 509–549 (1966).
20. Christiansen, R. L. & McKee, E. H. *Geol. Soc. Am. Mem.* **152**, 283–311 (1978).
21. Parsons, T., Thompson, G. A. & Sleep, N. H. *Geology* **22**, 83–86 (1994).
22. White, R. S. & McKenzie, D. P. *J. geophys. Res.* **94**, 7685–7730 (1989).
23. Richards, M. A., Duncan, R. A. & Courtillot, V. E. *Science* **246**, 103–107 (1989).
24. Duncan, R. A. & Richards, M. A. *Rev. Geophys.* **29**, 31–50 (1991).
25. Simpson, D. W. & Anders, M. H. *GSA Today* **2**, 118–121 (1992).
26. Fritz, W. J. & Sears, J. W. *Geology* **21**, 427–430 (1993).
27. Pelton, J. R. & Smith, R. B. *Science* **206**, 1179–1182 (1979).

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The most ancient African turtle

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ALTHOUGH turtles obviously differ greatly from other tetrapods in their shell, the turtle skull is so highly modified that turtle origins are difficult to resolve without evidence about the order of character acquisition. We report here the discovery of a turtle in the Early Jurassic Elliot Formation, South Africa, which extends the history of turtles in Africa by 60 million years, and reveals a previously unknown stage in the early evolution of turtles. This new skull is dominated by primitive characters held in common with the most primitive turtle known, the Late Triassic *Proganochelys* but, more notably, it is advanced in some important features. Previous turtle phylogenies suggest that most of the typical chelonian skull features evolved after turtles split into the two major groups, the Cryptodira and the Pleurodira. But this skull demonstrates that important cranial reorganization unexpectedly took place before the origin of the modern turtle groups. The reasons for this are unclear, but it may be related to the early evolution of hearing adaptations.

Order Testudines
Family Australochelyidae nov.
Australochelys gen. nov.

Type species. *Australochelys africanus*, new species.

Known distribution. Early Jurassic, Orange Free State, South Africa.

Etymology. *Australos*, Greek for south; *chelys*, Greek for turtle.
Diagnosis. As for species.

Australochelys africanus gen. et sp. nov.

Type specimen. Bernard Price Institute, University of the Witwatersrand, Johannesburg, South Africa, BP/1/4933; consists of a skull without lower jaws (Figs 1 and 2) and a fragment from the bridge area of the shell.

Locality. Bormansdrift, Clocolan District, Orange Free State, South Africa. Map sheet 1: 50,000 2827 CD Mekoatlengnek $28^{\circ} 57' 33'' \text{ S}$, $27^{\circ} 26' 05'' \text{ E}$ (ref. 1).

Horizon. Tritylodon Acme-Zone, (middle) Elliot Formation, Early Jurassic (ref. 2).

Collector. James W. Kitching, March 1980.

Etymology. *africanus*, for Africa.

Diagnosis. See ref. 3 for terminology. Primitive chelonian characters found only in *Proganochelys* and *Australochelys* within

Received 18 October 1993; accepted 17 March 1994.

1. Morgan, W. J. *Nature* **230**, 42–43 (1971).
2. Duncan, R. A. *Tectonophysics* **74**, 29–42 (1981).
3. Morgan, W. J. *Tectonophysics* **94**, 123–139 (1983).
4. Armstrong, R. L., Leeman, W. P. & Malde, H. E. *Am. J. Sci.* **275**, 225–251 (1975).
5. Pollitz, F. F. *Tectonics* **7**, 711–726 (1988).
6. Rodgers, D. W., Hackett, W. R. & Ore, H. T. *Geology* **18**, 1138–1141 (1990).
7. Pierce, K. L. & Morgan, L. A. in *Regional Geology of Eastern Idaho and Western Wyoming* (eds Link, P. K., Kuntz, M. A. & Platt, L. B.) 1–53 (Geol. Soc. Am. Mem. 179, Boulder, Colorado, 1992).
8. DeMet, C., Gordon, R. G., Argus, D. F. & Stein, S. *Geophys. J. Int.* **101**, 425–478 (1990).
9. Gripp, A. E. & Gordon, R. G. *Geophys. Res. Lett.* **17**, 1109–1112 (1990).
10. Suppe, J., Powell, C. & Berry, R. *Am. J. Sci.* **275-A**, 397–436 (1975).
11. Smith, R. B. & Arabasz, W. J. in *Neotectonics of North America* (eds Slemmons, D. B., Engdahl, E. R., Zoback, M. D. & Blackwell, D. D.) 185–228 (Geol. Soc. Am., Boulder, Colorado, 1991).
12. Geist, D. & Richards, M. *Geology* **21**, 789–792 (1993).
13. Zoback, M. L. & Thompson, G. A. *Geology* **6**, 111–116 (1978).
14. Scott, W. E., Pierce, K. L. & Hait, M. H. Jr. *Bull. seism. Soc. Am.* **75**, 1053–1066 (1985).
15. Anders, M. H., Geissman, J. W., Piety, L. A. & Sullivan, J. T. *J. geophys. Res.* **94**, 1589–1621 (1989).

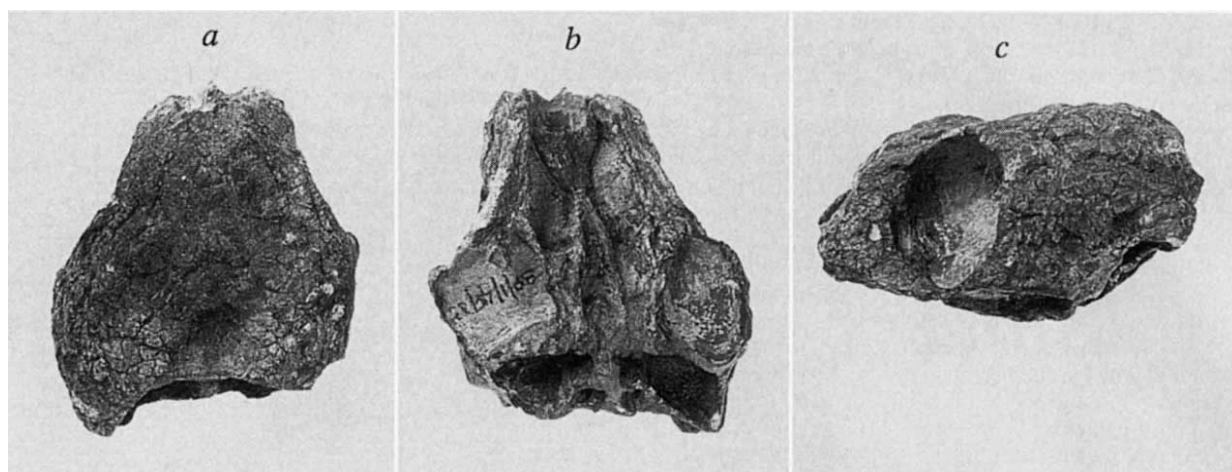


FIG. 1 *Australochelys africanus* gen. et sp. nov., Early Jurassic, Orange Free State, South Africa. BP/1/4933, type skull. a, Dorsal view; b, ventral view; c, left lateral view.

turtles but also found in primitive tetrapods: divided external nares; large interpterygoid vacuity as in *Proganochelys*; a recessed cavum tympani absent; lacrimal foramen present; recessus scalae tympani and fenestra perilymphatica absent; definitive foramen jugulare posterius absent; cultriform process present. Advanced chelonian characters found only in *Australochelys* and *Casichelydia* (cryptodires plus pleurodires): basiptyergoid articulation fused (Fig. 3b, 1'); stapes probably does not articulate directly with quadrate but may attach to a tympanic membrane supported by the acute posterior edge of the quadrate; distal end of opisthotic is covered by quadrate; middle ear region is partially enclosed laterally (Fig. 3b, 2'); canalis cavernosus is a well defined foramen; few or no palatal teeth; crista supraoccipitalis is a distinct vertical sheet of bone; temporal roof extends posteriorly over opisthotic (Fig. 3b, 3'). Characters unique to *Australochelys* (Figs 1 and 2): orbit larger in relative size than in any other turtle; lacrimal foramen at least three times larger than in *Proganochelys*; external nares elongate in contrast to all turtles; vomers highly arched dorsally, narrow posteriorly and very broad anteriorly in a unique configuration.

Turtles are an important group for morphological and molecular phylogenetic study because they have a good fossil record extending from the Late Triassic, and enough of their diversity has survived to the Recent⁴. Substantial structural and temporal gaps still exist between the most primitive known form, *Proganochelys* from the Late Triassic of Germany⁵, and the two major living groups, the Cryptodira and the Pleurodira. The new African turtle, *Australochelys*, helps bridge this gap with a combination of primitive tetrapod features and advanced features found in cryptodires and pleurodires.

The discovery of a turtle in the Elliot Formation of South Africa extends the range of chelonians in Africa from the Early Cretaceous⁶ back to the Early Jurassic, and it opens a new chapter in the history of turtles. The new African fossil (Figs 1 and 2) is a generally primitive turtle similar in many features to *Proganochelys*, the Late Triassic sister group to all other turtles⁵. However, the significance of the new form is that despite its similarities to *Proganochelys*, *Australochelys* also has advanced characters that indicate a sister group relationship to the *Casichelydia*^{4,5,7}, the group that consists of the Cryptodira and Pleurodira.

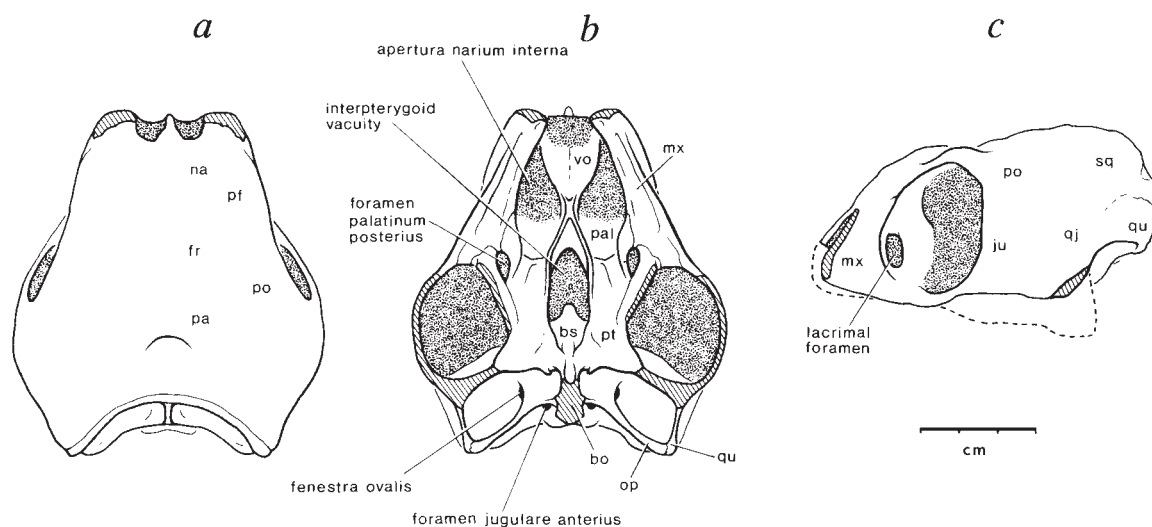


FIG. 2 *Australochelys africanus*, new genus and species, Early Jurassic, Orange Free State, South Africa. Partially restored type skull, a, Dorsal view; b, ventral view; c, left lateral view. bo, Basioccipital; bs basi-

sphenoid; fr, frontal; ju, jugal; mx, maxilla; na, nasal; op, opisthotic; pa, parietal; pal, palatine; pf, prefrontal; pt, pterygoid; qj, quadratojugal; qu, quadrate; sq, squamosal; vo, vomer.

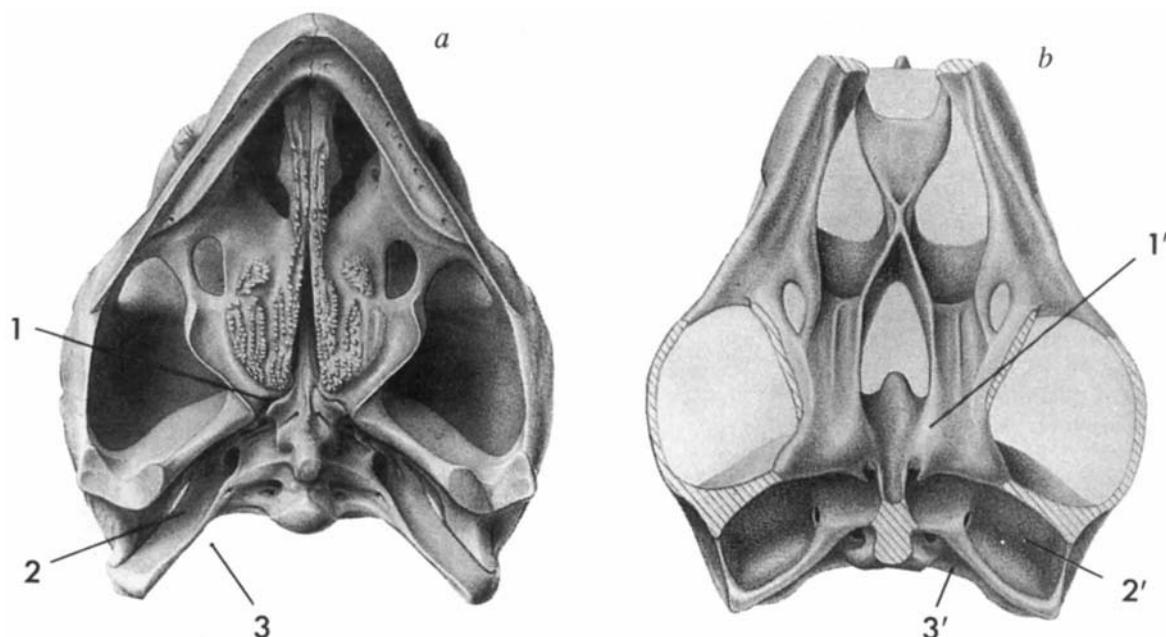


FIG. 3 a, *Proganochelys quenstedti*, Late Triassic, Germany (SMNS 16980, from ref. 5). b, *Australochelys africanus*, Early Jurassic, South Africa BP/1/4933. Ventral views showing three characters that are advanced in *Australochelys* and primitive in *Proganochelys*. 1, Movable

basipterygoid articulation; 1', fused basipterygoid articulation. 2, Unenclosed middle ear region; 2', middle ear region partially enclosed laterally. 3, Temporal roof does not extend posterior to opisthotic (not visible in ventral view); 3', temporal roof does extend posterior to opisthotic.

Australochelys and *Proganochelys* differ from all other turtles (the Casichelydia) in retaining these primitive tetrapod characters (Fig. 3): interpterygoid vacuity, cultriform process, lacrimal foramen, divided external nares, and a relatively open middle ear region (cavum acustico-jugulare)^{3,5}. The principal features of *Australochelys* that are advanced over *Proganochelys* involve the attachment of the braincase to the rest of the skull, an area that is characteristically modified in the Cryptodira and Pleurodira. In *Proganochelys* (Fig. 3a) and primitive tetrapods the basipterygoid articulation, the contact between the braincase and the palate, is a moveable joint and the associated cranio-quadrates space containing the lateral head vein is an open fissure⁵. In *Australochelys* (Fig. 3b) and all other turtles (the Casichelydia) the joint is fused and the surrounding bones are sutured to make it immovable, and the cranio-quadrates space is ossified and reduced to a foramen, the canalis cavernosus³. All other turtles that have the braincase and skull fused also have other specializations that further brace the braincase and enclose the

ear region in bone. *Australochelys* shows that the first step in the evolution of this complex was the change from a moveable basipterygoid articulation to a sutured contact that did not allow movement. This can even be hypothesized as a necessary structural precursor that prevented braincase movement within the skull allowing further ossification of the middle ear and the evolution of the jaw muscle trochleas typical of the living turtle groups.

The identification of a Late Triassic pleurodire, *Proterochersis*^{4,8}, shows that both its contemporary *Proganochelys* and the slightly younger *Australochelys* were already 'living fossils' (Fig. 4). The discovery of *Australochelys* shows that despite the origin of the modern turtle groups in the Triassic, more primitive chelonians persisted and were widespread. Recent discoveries of turtles in the Late Triassic/Early Jurassic of North and South America and Asia, as well as Africa, further document a picture of a worldwide radiation in the early history of turtles.

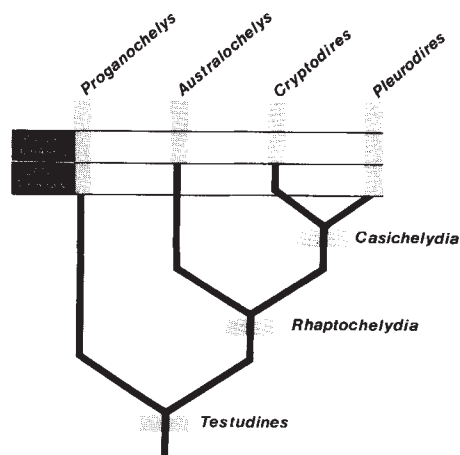


FIG. 4 Cladogram showing relationships of *Australochelys* to other turtles. Stratigraphic ranges of terminal taxa indicated to show that the presence of the phylogenetically more advanced pleurodires in the Late Triassic makes *Australochelys* from the Early Jurassic a 'living fossil' among its contemporaries. *Testudines*, see ref. 1 for diagnosis. *Rhaptochelydia*, new taxon, raptors, Greek for 'sewn' in allusion to the sutured basicranial articulation, chelys, Greek for turtle. Diagnosis: sutured basicranial articulation; middle ear enclosed laterally; temporal roof extends posterior to opisthotic; paroccipital process of opisthotic tightly sutured to braincase. *Casichelydia*, revised diagnosis: lacrimal foramen absent, interpterygoid vacuity small or absent, middle ear region enclosed ventrally, definitive (recessed) cavum tympani present, foramen jugulare posterius defined by bone, recessus scalae tympani and fenestra perilymphatica defined by bone.

In contrast to the presumably partially aquatic, *Proganochelys*⁵, *Australochelys* was found in a deposit with terrestrial vertebrates that is thought to represent an arid environment¹, suggesting that by the Early Jurassic turtles were ecologically diverse. □

Received 10 January; accepted 11 March 1994.

1. Kitching, J. W. & Raath, M. A. *Palaeont. afr.* **25**, 111–125 (1984).
2. Olsen, P. E. & Galton, P. M. *Palaeont. afr.* **25**, 87–110 (1984).
3. Gaffney, E. S. *Bull. Am. Mus. nat. Hist.* **164**, 65–376 (1979).
4. Gaffney, E. S. & Meylan, P. A. *The Phylogeny and Classification of the Tetrapods* Vol. 1 *Amphibians, Reptiles, Birds* (ed. Benton, M. J.) 157–219 (Systematics Assoc. Special vol. No. 35A, Clarendon, Oxford, 1988).
5. Gaffney, E. S. *Bull. Am. Mus. nat. Hist.* **194**, 1–263 (1990).
6. De Broin, F. *Ecosystèmes Continentaux du Mésozoïque* (ed. Taquet, P.) 39–46 (Mem. Soc. Geol. Fr., Paris, 1980).
7. Gaffney, E. S. *Bull. Am. Mus. nat. Hist.* **155**, 387–436 (1975).
8. Fraas, E. *Jahresh. Ver. Vaterland Naturk. Württemberg* **80**, 13–30 (1913).

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Mycorrhizae alter quality and quantity of carbon allocated below ground

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PLANTS and soils are a critically important element in the global carbon-energy equation. It is estimated that in forest ecosystems over two-thirds of the carbon is contained in soils and peat deposits¹. Despite the importance of forest soils in the global carbon cycle, fluxes of carbon associated with fundamental processes and soil functional groups are inadequately quantified, limiting our understanding of carbon movement and sequestration in soils. We report here the direct measurement of carbon in and through all major pools of a mycorrhizal (fungus-root) coniferous seedling (a complete carbon budget). The mycorrhizal symbiont reduces overall retention of carbon in the plant-fungus symbiosis by increasing carbon in roots and below-ground respiration and reducing its retention and release above ground. Below ground, mycorrhizal plants shifted allocation of carbon to pools that are rapidly turned over, primarily to fine roots and fungal hyphae, and host root and fungal respiration. Mycorrhizae alter the size of below-ground carbon pools, the quality and, therefore, the retention time of carbon below ground. Our data indicate that if elevated atmospheric CO₂ and altered climate stressors alter mycorrhizal colonization in forests, the role of forests in sequestering carbon could be altered.

Ectomycorrhizae are one form of fungus-root symbioses whose morphological characteristics include a root enclosed in a sheath of fungal tissue and a complex network of hyphae between root epidermal and cortical cells that extends into the soil^{2,3}. The intimate morphology extends the surface area for carbon and nutrient exchange between symbionts, and increases the area in contact with soil compared with the non-mycorrhizal condition. Until now, complete carbon budgets for any mycorrhizal symbiosis were unobtainable because of the lack of suitable measuring techniques⁴. Previous carbon allocation models were based on differences between entire non-inoculated and mycorrhizal seedlings, and not on calculations using direct measures of fungal processes and respective fungal mass. Estimates of carbon retention and respiration by hyphae of the vesic-

TABLE 1 Fraction dry weights, and relative activity of fractions adjusted to 10⁵ Bq total ¹⁴C assimilation by needles

Fraction	Non-inoculated		Ectomyc.	
	Dry weight (mg)		Relative activity (Bq ¹⁴ C mg ⁻¹)	
Total plant	2,080	1,878*		
Above ground				
Total	940	868*	50.0	44.0
			Respiration	44.0
			Remaining	22.4
Bud and stem	266	238	20.0	22.4
Needles	674	630	27.0	27.0
Below ground				
Total	1,137	1,010	15.3	22.1**
Roots (host)	1,137	910***	15.3	19.8
Active hyphae†	n.p.	100***	n.p.	40.1***
			Respiration	22.1**
			Remaining	17.7
Coarse roots	936	741**	14.6	17.7
Fine roots (host)	201	169	16.9	23.4*
Active hyphae	n.p.	100***	n.p.	32.8***

Values are means of numbers that were calculated using individual seedling data of ¹⁴C allocation to fractions and the respective fraction dry weight. The individual seedling relative activity value was then standardized to 10⁵ Bq total radioisotope assimilation by needles. All statistical conventions are as in Fig. 2. n.p., Not present.

† See Fig. 2 legend for estimates of hyphal mass in ectomycorrhizal root tips.

ular arbuscular mycorrhizal (VAM) symbiosis range from 4% to 20% (refs 5–8). Ectomycorrhizal hyphal respiration was estimated as 30% of below-ground respiration⁹. However, specific rates were not calculated because hyphal biomass was not quantified.

We studied the ectomycorrhizal association between ponderosa pine (*Pinus ponderosa* Laws.) seedlings and *Hebeloma crustuliniforme* (Bull.: St. Amans) Quel that developed in root-mycosoms (Fig. 1, and refs 10, 11). Root-mycosoms allowed us to measure carbon fluxes through a portion of intact hyphae while symbiotic integrity was maintained. Photosynthesis of shoots and respiration of all structural fractions were first measured, and seedlings were then used to determine allocation and respiration of recently fixed ¹⁴C (ref. 10). Fluxes from host and fungus were calculated after direct measurements were made on the fungus and various host fractions. A complete carbon budget was then constructed using respective fraction mass.

When all ¹⁴C retention values were summed, mycorrhizal seedlings retained 39.8% of the ¹⁴C fixed by needles and non-inoculated seedlings retained 41.3% (Fig. 2). The small amount of active fungal mass (5% of total mycorrhizal seedling dry weight) increased ¹⁴C allocation below ground by about 23% compared with non-inoculated seedlings. The fungus received about 7% of the total ¹⁴C fixed. As the fungus contributed a small amount to total seedling dry weight, our data suggest that the fungus did not receive an inordinate amount of carbon (high carbon cost to the host has been a widespread speculation). Sixty per cent of the ¹⁴C allocated to the fungus, or 4.3% of total ¹⁴C assimilated, was respired by the fungus. Hyphal respiration represented 19.4% of total below-ground respiration. The hypothesis that a small amount of fungus has a substantial effect on carbon allocation within mycorrhizal plants^{7,12,13,14} is supported by our data.

We calculated that bacterial respiration was less than 2% of hyphal respiration or 0.2% of total below-ground respiration, and ¹⁴C release as root exudation was negligible (Fig. 2). Therefore, these fluxes were considered insignificant in our budget and are included with the respective pool.

Total above-ground ¹⁴C respiration by mycorrhizal seedlings was about 10% less than non-inoculated seedlings, whereas below-ground respiration of mycorrhizal seedlings was about 35% greater. Above-ground ¹⁴C allocation values between

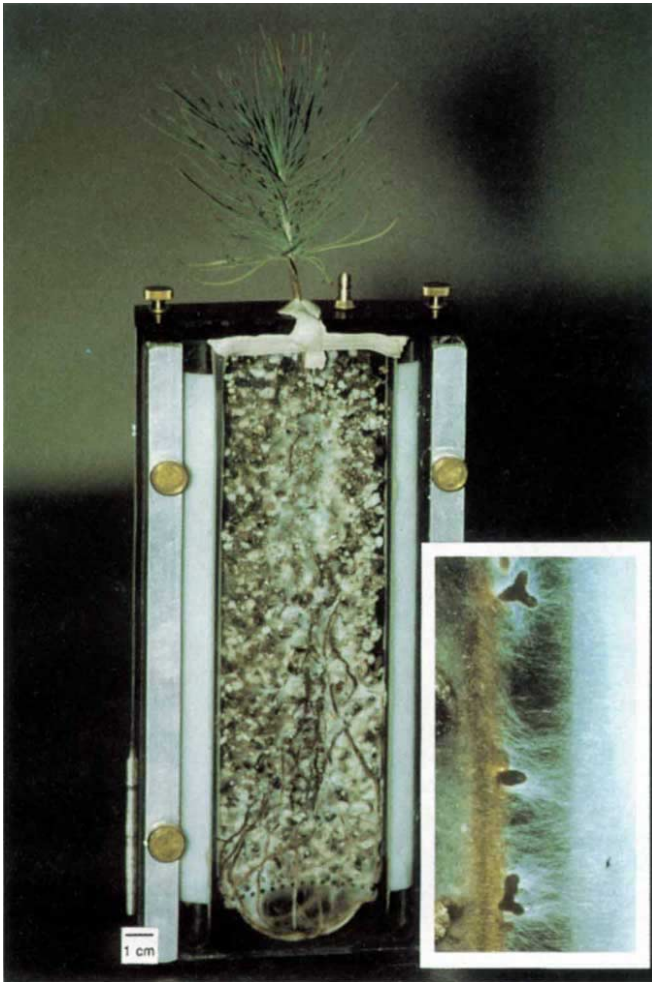
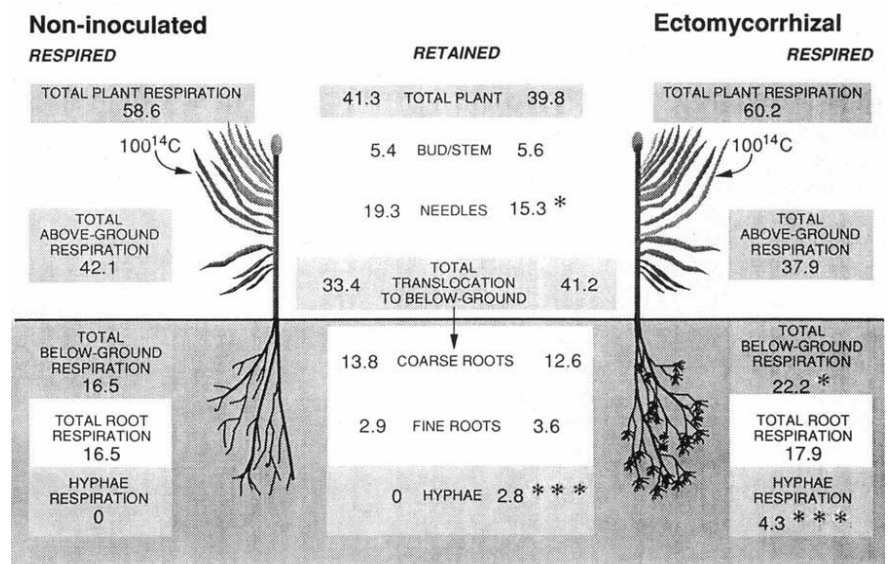


FIG. 1 Ponderosa pine (*Pinus ponderosa* Laws.) seedling (6–7-month-old) in symbiosis with *Hebeloma crustuliniforme* in a root-mycocosm. Insert shows extramatrical hyphae emanating from ectomycorrhizae and passing from the root compartment to one of the fungal compartments.

METHODS. Isolate S-260 of *H. crustuliniforme* (USDA Forest Sciences Laboratory, Corvallis, Oregon, USA; obtained from Mycorrh Tech Inc., Pittsburgh, Pennsylvania, USA) and ponderosa pine seedlings were grown in root-mycocosms (2.5 cm thick, 11.5 cm wide and 23 cm long) in the growth chamber as previously described^{10,11}. Hyphae passed from the root compartment into the fungal compartments through the gap created between the glass face plate and the wall separating the two types of compartments. Hyphae in the fungal compartment grew onto glass-fibre filter paper backed by Nitran membrane and rockwool. Seedlings were prepared for ¹⁴C pulse-chase and gas exchange experiments by removing the glass face plate and applying hydrated lanolin to the surfaces of the black plastic block that are in contact with the glass (including over the hyphae passing between compartments) so that each compartment was hermetically sealed allowing the gas exchange of each symbiont to be independently measured. A completely randomized design was used to assign eight seedlings to two treatments, with and without fungus. The experiments were replicated once in time ($n=8$) and all dependent data were subjected to analysis of variance (ANOVA) to test for fungal treatment effects¹⁹. Residual analyses indicated that the usual normality and homogeneity of variance assumptions were satisfied²⁰.

FIG. 2 Flow of ¹⁴C (Bq ¹⁴C allocated during the chase period per 100 Bq ¹⁴C assimilated by needles) through seedlings. Differences between fungal treatments significant at: * $\alpha=0.15$, ** $\alpha=0.10$, *** $\alpha=0.05$.

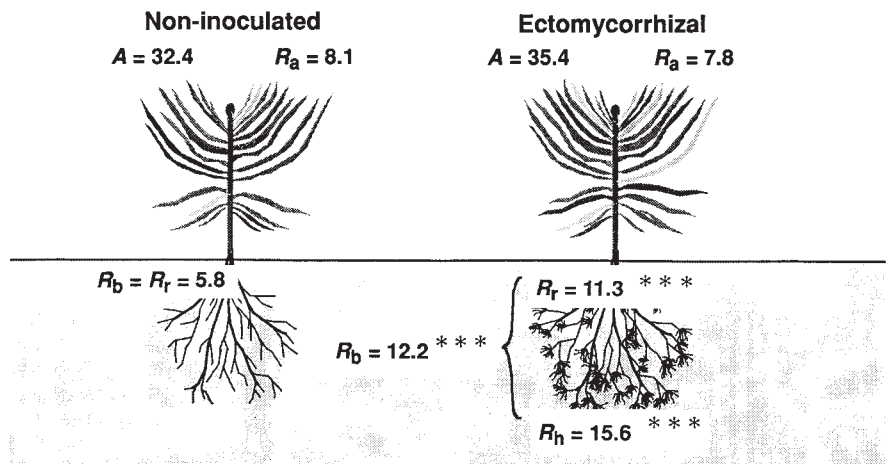
METHODS. Seedlings were labelled for 1 h with 1,850 kBq NaH ¹⁴CO₃, respiration gases were collected during a 72 h translocation period, seedlings harvested, and tissue and respiration samples analysed for radioisotope^{10,14}. Below-ground plant parts were classified as fine roots (≤ 1 mm), mycorrhizae, or coarse roots. Total and active hyphal mass in root compartments of experimental seedlings, and active hyphae in the fungal compartments of a matched set of root-mycocosms, were estimated using light microscopy and fluorescein diacetate (FDA)²¹. Roughly 6% and 100% (by weight) of hyphae in the root and fungal compartments took up FDA, respectively. Total hyphal mass in the fungal compartments was determined gravimetrically using the difference in mass of the filter paper before installation and at harvest. Hyphal mass of mycorrhizal seedlings was calculated using the active mass in root and fungal compartments, and of the Hartig net and sheath of the mycorrhizae (30% of the dry weight of an ectomycorrhiza, ref. 3, p. 128). Specific rates in the pulse-chase and gas exchange experiments were based on direct measures for fungus-only and fungus-plus-host fractions of inoculated seedlings, and for the host-only fractions of non-inoculated seedlings. Host root rates in symbiosis were inferred. No active bacteria were found (FDA staining²¹) in the fungal compartments and an average of 2 mg were found in the root compartment. We



assumed similar hyphal and bacterial respiration rates to calculate the bacterial contribution to below-ground respiration. Below-ground compartments were washed with 0.1M KCl to collect extractable ¹⁴C-labelled exudates¹⁴. Values were calculated by adjusting total ¹⁴C assimilation for each seedling type to 100 Bq, then the proportional adjustment was applied to each fraction.

FIG. 3 Rates of respiration and net assimilation of CO₂ in non-inoculated *Pinus ponderosa* seedlings and seedlings colonized with *Hebeloma crustuliniforme*. Statistical conventions as in Fig. 2. Units are: Above-ground, A, assimilation, nmol CO₂ per g dry weight top per s; R_a, respiration, nmol CO₂ per g dry weight top per s; Below-ground, R_r, respiration of roots, nmol CO₂ per g dry weight per s; R_h, respiration of hyphae, nmol CO₂ per g dry weight active hyphae per s; R_b, total below-ground respiration, nmol CO₂ per g dry weight roots and hyphae per s.

METHODS. Assimilation and respiration were measured using a closed-loop infrared gas analyser (IRGA) 24 ± 1 °C (ref. 10). Above-ground respiration was measured after steady-state conditions were achieved in the dark using a shade cloth. Below-ground respiration was measured after removing the cuvette from the IRGA and connecting the inflow and outflow lines directly to hose barbs of the below-ground compartments to form a closed loop. Each below-ground compartment was first scrubbed with CO₂-free air to achieve a standard CO₂ concentration similar to ambient



seedling types were similar. However, there was a trend ($P = 0.15$) towards lesser retention by needles in the mycorrhizal seedlings, as noticed previously¹⁴.

Because allocation is also a function of fraction size, we standardized the allocation values by dry weight to make direct comparisons of relative fraction activity (Table 1). In general, the relative activity of the below-ground fraction of mycorrhizal seedlings was greater than non-inoculated seedlings. Specifically, the highest relative activities below ground occurred in pools of mycorrhizal seedlings that are rapidly turned over and returned to the atmosphere, for example hyphae and fine roots. Fungal hyphae had the greatest relative activity of all below-ground fractions.

Hyphae had the greatest respiration rates of any seedling fraction; *H. crustuliniforme* respired 15.6 nmol CO₂ per g dry weight active hyphae per second (Fig. 3). These are the first reported rates of respiration of ectomycorrhizal hyphae in symbiosis using fungal mass that was directly quantified.

The total below-ground respiration rate of mycorrhizal seedlings was 2.1 times the rate of non-inoculated seedlings (Fig. 3). The greater rate is due to at least three factors. First, the hyphae had the highest respiration rate, as noted above. Second, colonized roots had inherently higher calculated respiration rates compared with non-inoculated roots, indicating a 'colonization respiration' for ectomycorrhizal host roots. Colonization respiration has been estimated only for VAM-colonized roots¹⁵ and is demonstrated for tissue infected by pathogens ('infection respiration')¹⁶. Third, mycorrhizal seedlings had a greater percentage of active fine roots (fine root/coarse root weight ratio: 1.32 versus 1.16 in mycorrhizal and non-inoculated seedlings, respectively). Collectively, the three processes contribute to the more rapid release of photosynthetically fixed carbon in mycorrhizal plants.

There was an indication that mycorrhizal seedlings accumulated less biomass than non-inoculated seedlings ($P = 0.15$; Table 1), resulting from slightly less retention in seedling tops, a shift in allocation from coarse roots to fine roots in mycorrhizal seedlings, and the production of hyphae. Conifers in symbiosis with *H. crustuliniforme* accumulate less mass despite similar photosynthetic rates compared with non-inoculated seedlings which may be related to the carbon allocated to form an extensive hyphal network outside the root¹³. Our data indicate that to account fully for the differences in mass between seedling types, the respiration of the fungus and the colonization respiration of host roots must be considered. These fluxes illustrate the import-

ance of symbionts in below ground and total plant carbon dynamics, even when the symbiont mass is relatively small.

Small changes in carbon use in plants can result in large shifts in carbon cycling at larger scales. Changes in the colonization of roots by mycorrhizal fungi, or in the proportional allocation of carbon to form coarse and fine roots may affect the sequestration of carbon in ecosystems. Factors such as elevated CO₂ also increase carbon incorporation into below-ground pools with short residence times such as fine roots^{17,18}. Regardless of how elevated CO₂ influences mycorrhizal colonization and activity, the contribution of mycorrhizal systems to the total carbon budget of forests must be evaluated if accurate estimates of carbon fluxes at larger scales are to be made. For our data to be of most use in models, adjustments may be needed for soil temperature, precipitation, seasonal root and mycorrhizae formation and growth, and the diversity of plant species/fungal species associations. Investigations are underway to characterize partitioning of carbon below ground under various future climates to improve model predictions of carbon sequestration¹. □

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- Dixon, R. K. et al. *Science* **263**, 185–190 (1994).
- Allen, M. F. *The Ecology of Mycorrhizae* 184 (Cambridge University Press, New York, 1991).
- Harley, J. L. & Smith, S. E. *Mycorrhizal Symbiosis* 483 (Academic, New York, 1983).
- Jakobsen, I. in *Methods of Microbiology*, Vol. 23, *Techniques of the Study of Mycorrhiza* (eds Norris, J. R., Read, D. J. & Varma, A. K.) 149–180 (Academic, New York, 1991).
- Paul, E. A. & Kucey, R. M. N. *Science* **213**, 473–474 (1981).
- Kucey, R. M. N. & Paul, E. A. *Soil Biol. Biochem.* **14**, 407–412 (1981).
- Jakobsen, I. & Rosendahl, L. *New Phytol.* **115**, 77–83 (1990).
- Peng, S., Eissenstat, D. M., Graham, J. H., Williams, K. & Hodge, N. C. *Plant Physiol.* **101**, 1063–1071 (1993).
- Söderström, B. & Read, D. J. *Soil Biol. Biochem.* **19**, 231–236 (1987).
- Andersen, C. P. & Rygielwicz, P. T. *Environ. Pollut.* **73**, 217–244 (1991).
- Rygielwicz, P. T., Miller, S. L. & Durall, D. M. *Pl. Soil* **109**, 281–284 (1988).
- Rousseau, J. V. D. & Reid, C. P. P. *A. Sci. For.* **46** (suppl.) 715–717 (1989).
- Dosskey, M. G., Linderman, R. G. & Boersma, L. *New Phytol.* **115**, 269–274 (1990).
- Miller, S. L., Durall, D. M. & Rygielwicz, P. T. *Tree Physiol.* **5**, 239–249 (1989).
- Bass, R., van der Werf, A. & Lambers, H. *Pl. Physiol.* **91**, 227–232 (1989).
- Agrios, G. N. *Plant Pathology* 3rd edn 90–93 (Academic, New York, 1988).
- Norby, R. J., O'Neill, E. G. & Luxmoore, R. J. *Pl. Physiol.* **82**, 83–89 (1986).
- Norby, R. J., Gunderson, C. A., Wulfschlegel, S. D., O'Neill, E. G. & McCracken, M. K. *Nature* **357**, 322–324 (1992).
- SAS Institute Inc. *SAS/STAT User's Guide*, Version 6, 4th edn, Vol. 2, 891–996 (Cary, North Carolina, 1989).
- Montgomery, D. C. *Design and Analysis of Experiments* 85–98 (Wiley, New York, 1976).
- Lodge, D. J. & Ingham, E. R. *Agric. Ecosys. Environ.* **34**, 131–144 (1991).

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Nitric oxide mediates network oscillations of olfactory interneurons in a terrestrial mollusc

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THE interneuronal messenger nitric oxide^{1,2} (NO) may play a central role in the processing of olfactory information³. Several circuit elements in the mammalian olfactory bulb contain NO synthase⁴ or its functional equivalent, NADPH diaphorase⁵⁻⁷. The effects of NO on cellular excitability or circuit dynamics in the olfactory bulb are unknown, although NO effects on other rhythmic cells⁸ and circuits⁹ have been described. I have studied the role of NO in central olfactory processing using the procerebral (PC) lobe, the major central site of odour processing in terrestrial molluscs^{10,11}. As in the mammalian olfactory bulb during odour stimulation, the basic dynamics of electrical activity in the molluscan PC lobe is an oscillation¹²⁻¹⁴. Here I report an obligatory role for NO in the oscillatory dynamics of the PC lobe of *Limax maximus*. Nitric oxide mediation of the olfactory oscillation may relate to the highly developed odour sensitivity and odour-learning ability of *Limax*^{15,16}.

The coherent oscillation in field potential of the PC lobe of *Limax* arises from interactions between about 10^4 bursting, putatively glutaminergic cells, and about 10^5 non-bursting cells that receive periodic inhibitory postsynaptic potentials (i.p.s.ps) from the bursting cells^{12,14,17}. Intracellular recordings from bursting and non-bursting cells during NO application clarified how NO affects the field potential oscillation by modifying the activity patterns of bursting and non-bursting cells.

The *Limax* PC lobe contains neurons that show NADPH diaphorase staining (Fig. 1a), as previously shown in *Helix* PC lobe¹⁸. The diaphorase-positive PC cells are scattered throughout the cell body layer with higher density in the distal half. Five per cent of PC cells freshly isolated on polylysine-coated coverslips show diaphorase staining (20 of 386 cells, 3 preparations). Diaphorase-positive PC cells are presumptive nitric oxide synthase-containing, NO-producing cells. Diaphorase staining of cells in other regions of the *Limax* central nervous system (CNS) has also been reported¹⁹.

The necessity and sufficiency of NO for generation of the field

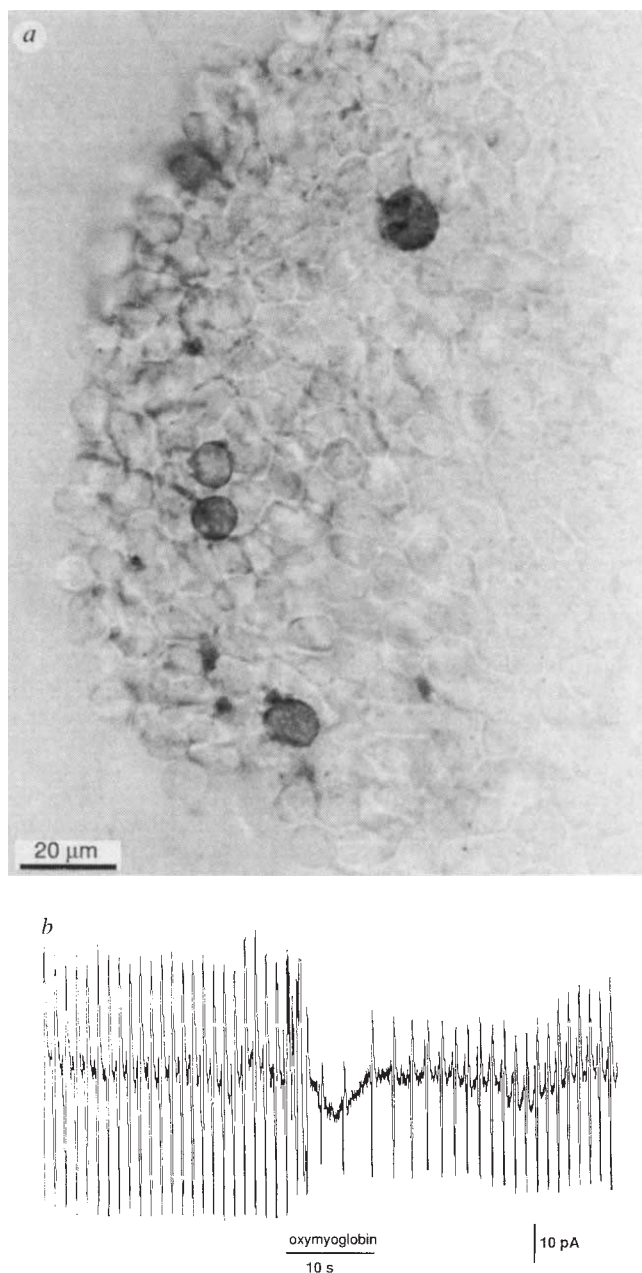


FIG. 1 Oxymyoglobin but not metmyoglobin slows the PC oscillation. a, Diaphorase-stained neurons in the cell body region of the PC lobe. b, A bolus of 2 mM oxymyoglobin transiently slows the PC oscillation. c, Plot of the PC oscillation frequency throughout the response shown in b. d, Mean responses ($\pm$ s.e.m.) to repeated interspersed applications of oxy- ($N=14$) and metmyoglobin ($N=13$) to 5 preparations. Oxymyoglobin significantly depressed the PC oscillation frequency whereas the response to metmyoglobin is not significantly different from control rate.

METHODS. PC cells or lobes were exposed to NADPH-nitroblue tetrazolium solution then fixed in 4% paraformaldehyde, rinsed and mounted in glycerol. PC lobes for electrophysiology were isolated by fine dissection from the cerebral ganglia, desheathed and placed in a chamber with multiple sources of perfusion solution. Alternatively, a bolus of drug solution was added to the chamber perfusion inlet and responses compared to those elicited by an equal volume of saline, which typically elicited little or no response. Field potential recording from the PC neuropil was made with a saline-filled micropipette of 3–5 μm tip opening ($R < 1$ MΩ). After current-to-

voltage conversion (list EPC-7) and amplification with band-pass filtering (0.3–10 Hz) (PAR 113), signals were digitized and stored on a Mac II Apple computer.

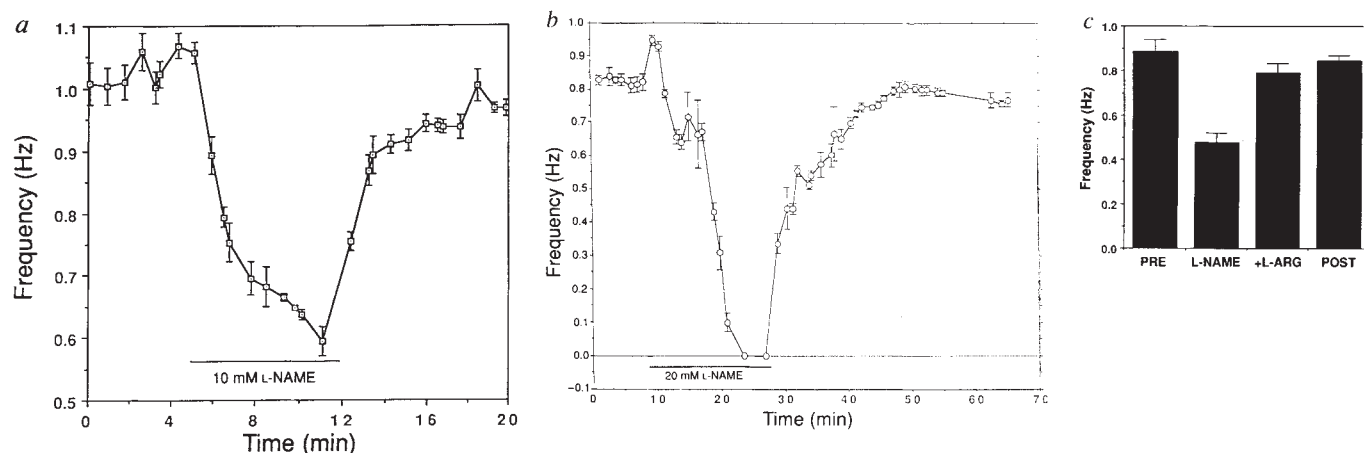


FIG. 2 A nitric oxide inhibitor slows and can stop the PC oscillation. *a*, Reduction in PC oscillation frequency elicited by 7-min exposure to 10 mM L-NAME. Mean values ($\pm$ s.d.) of 10 cycle samples of the oscillation frequency taken every 50 s are shown. *b*, An 18-min exposure

to 20 mM L-NAME stops the oscillation, which recovers promptly after washout. *c*, The reduction in PC oscillation frequency caused by 10 mM L-NAME is reversed by addition of 5 mM L-arginine. Data (mean $\pm$ s.d.) from 4 trials on 3 preparations are shown.

potential oscillation of the PC lobe was assessed by applying agents known to alter tissue levels of NO and recording the oscillation. The necessity of NO was tested with two treatments that reduce NO levels, and the sufficiency of NO was tested by three treatments that increase NO levels.

Extracellular application of the NO scavenger oxymyoglobin to the PC lobe caused a reduction in frequency of the oscillating field potential (Fig. 1*b, c*). Results from a series of oxymyoglobin applications ($N=14$), compared with an interspersed series of metmyoglobin applications ($N=13$) to 5 preparations, are shown in Fig. 1*d* (mean $\pm$ s.e.m.). Oxymyoglobin, but not metmyoglobin, significantly depressed PC oscillation frequency (unpaired, d.f. = 26, $t=6.59$, $P<0.01$). Maintained application of 5–10 mM oxymyoglobin to PC lobes from 0.1-g slugs stops the PC oscillation reversibly (6 trials, 3 preparations) (data not shown).

The nitric oxide synthase inhibitor L-N^G-nitroarginine methyl ester (L-NAME) also reduced the frequency of the field potential oscillation. The response to a 7-min application of 10 mM L-NAME is shown in Fig. 2*a*. Combining data from 5 preparations tested with 10 mM L-NAME and 5 preparations tested with 20 mM L-NAME, the average maximum reduction after 7 min was $36 \pm 2\%$ (mean $\pm$ s.e.m.). More prolonged exposure to 20 mM, but not 10 mM, L-NAME stopped the oscillation rever-

sibly (Fig. 2*b*). Complete, reversible suppression of the oscillation was seen in 10 trials on 5 lobes. The effect of 10 mM L-NAME on the PC oscillation frequency is reversed by adding 5 mM L-arginine. Data from 4 trials in 3 preparations are shown in Fig. 2*c*. L-NAME significantly reduced the oscillation frequency relative to the pretreatment level (d.f. = 6, $t=11.03$, $P<0.01$), whereas addition of 5 mM L-arginine returned the oscillation frequency to control levels. To test the possibility that mM concentrations of L-NAME slow the PC oscillation by blocking cholinergic receptors²⁰, atropine and curare (0.1 mM) were applied while measuring the PC oscillation frequency. In 5 trials on 4 preparations, the ratio of oscillation frequency during drug treatment to oscillation frequency during pre-drug control period was 1.06 for atropine and 0.99 for curare. Taken together, the results with a haem protein NO scavenger and an NO synthase inhibitor show that NO is necessary for generation of the PC oscillation.

To test whether exogenous NO can alter the PC oscillation frequency, a series of compounds known to increase tissue levels of NO were used. The NO-releasing compound diethylamine/NO ($(C_2H_5)_2N[N(O)NO]Na^+$ (DEA/NO) is one of a class of nucleophile/NO adducts that are stable as solids and release NO spontaneously in aqueous solution^{21–23}. DEA/NO generates 1.5 mol NO per mol starting material, with a half-life of 16 min

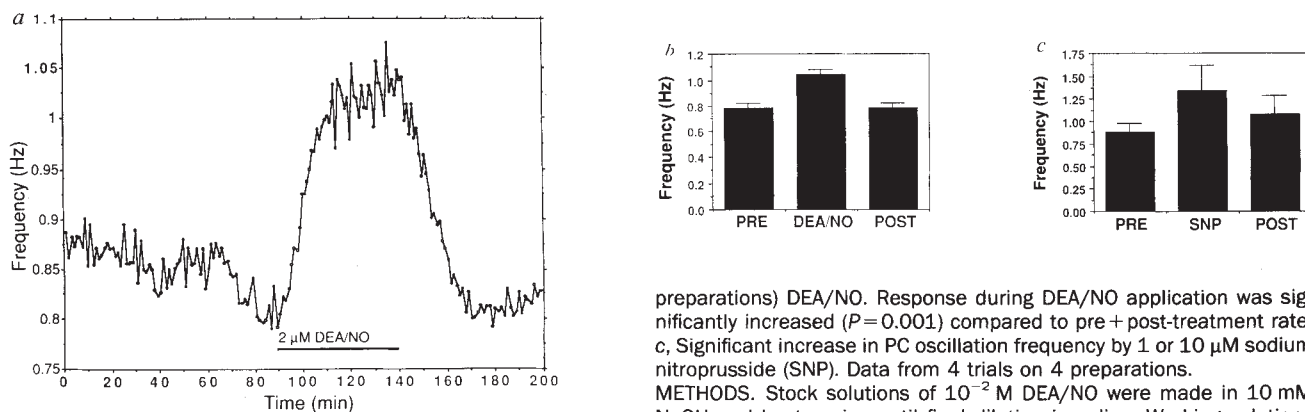


FIG. 3 NO liberation by DEA/NO increases the PC oscillation frequency. *a*, DEA/NO causes an increase in the frequency of the PC oscillation. *b*, Mean ($\pm$ s.d.) increase in oscillation frequency by a series of 50-s applications of 2 μ M (4 trials, 2 preparations) or 4 μ M (4 trials, 2

preparations) DEA/NO. Response during DEA/NO application was significantly increased ($P=0.001$) compared to pre + post-treatment rate. *c*, Significant increase in PC oscillation frequency by 1 or 10 μ M sodium nitroprusside (SNP). Data from 4 trials on 4 preparations. METHODS. Stock solutions of 10^{-2} M DEA/NO were made in 10 mM NaOH and kept on ice until final dilution in saline. Working solutions were used within a few minutes of final dilution. Control solutions were left at room temperature for 8 h to allow complete elimination of NO. SNP solutions were fresh and protected from light. Control SNP solutions kept for 20 h in ambient illumination had no effect.

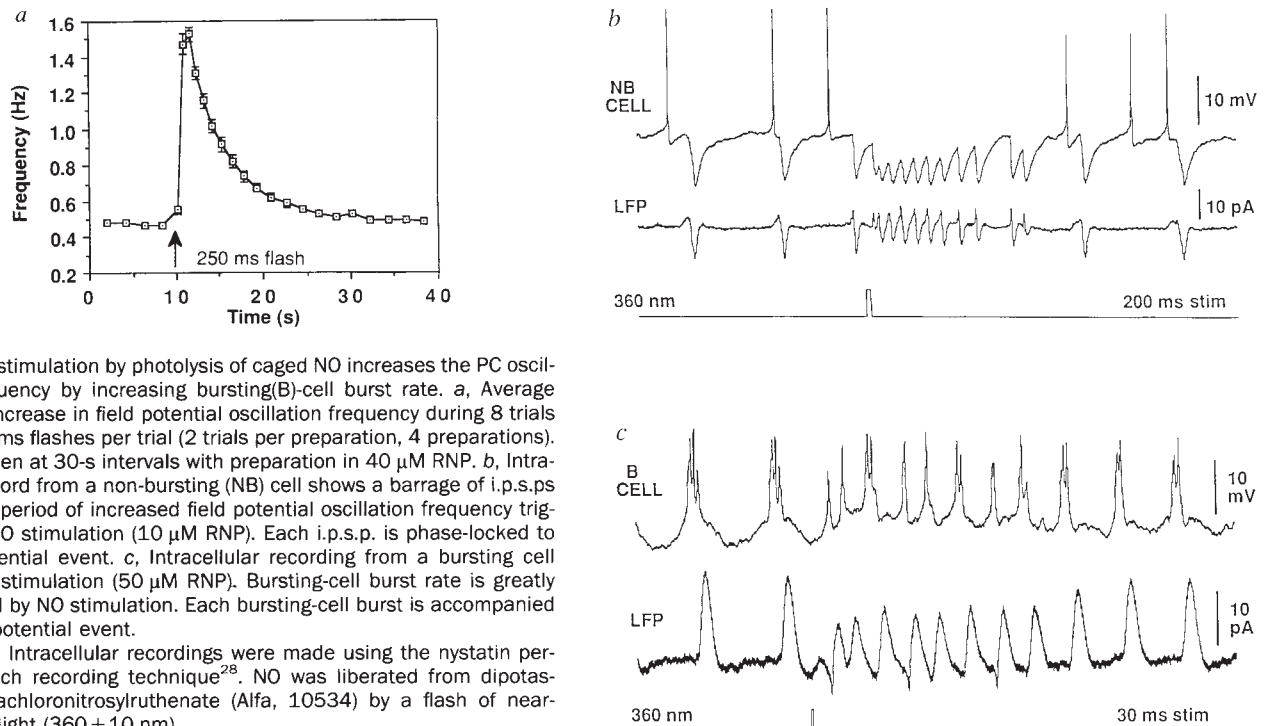


FIG. 4 NO stimulation by photolysis of caged NO increases the PC oscillation frequency by increasing bursting(B)-cell burst rate. **a**, Average ($\pm$ s.e.m.) increase in field potential oscillation frequency during 8 trials of six 250-ms flashes per trial (2 trials per preparation, 4 preparations). Flashes given at 30-s intervals with preparation in 40 μ M RNP. **b**, Intracellular record from a non-bursting (NB) cell shows a barrage of i.p.s.ps during the period of increased field potential oscillation frequency triggered by NO stimulation (10 μ M RNP). Each i.p.s.p. is phase-locked to a field potential event. **c**, Intracellular recording from a bursting cell during NO stimulation (50 μ M RNP). Bursting-cell burst rate is greatly augmented by NO stimulation. Each bursting-cell burst is accompanied by a field potential event.

METHODS. Intracellular recordings were made using the nystatin perforated-patch recording technique²⁸. NO was liberated from dipotassium pentachloronitrosylruthenate (Alfa, 10534) by a flash of near-ultraviolet light (360 ± 10 nm).

at 22 °C in pH 7.4 buffer²⁴. Application of 2 μ M DEA/NO for 50 s produced a significant increase in frequency of the PC oscillation (Fig. 3a), whereas a solution used 4 h after NO generation ended was without effect (data not shown). Grouped data for 2 μ M (4 trials, 2 preparations) and 4 μ M (4 trials, 2 preparations) DEA/NO showed a $32 \pm 3\%$ increase in frequency (Fig. 3b). A second NO generator, sodium nitroprusside, also increased the frequency of the PC oscillation (Fig. 3c) ($52 \pm 4\%$ increase, 4 trials, 4 preparations, 1–10 μ M). Both DEA/NO and SNP increased the PC oscillation frequency significantly.

The caged NO compounds ruthenium nitrosyl trichloride and ruthenium nitrosyl pentachloride (RNP) can be photolysed with near-ultraviolet light to liberate NO in aqueous solution^{25,26}. Preparations bathed in 40 μ M RNP showed normal PC oscillations in the absence of illumination. The average change in field potential oscillation frequency caused by 8 trials of six 250-ms flashes at 30-s intervals per trial (4 preparations) is shown in Fig. 4a (mean $\pm$ s.e.m.). Light flashes delivered in the absence of RNP produced no effect on field potential oscillation frequency. As a control for possible stimulatory effects of photoproducts other than NO, the PC was exposed to a 10 μ M RNP solution, previously irradiated so as to reduce the RNP concentration by more than 95% and left for sufficient time to allow complete release of NO. The previously irradiated RNP solution had no effect in the dark, and eliminated the flash-evoked acceleration of the oscillation (7 trials, 2 preparations).

Photolysis of caged NO increased the frequency of the field potential oscillation and affected activity in bursting and non-bursting cells in opposite ways. Intracellular recordings from non-bursting cells before illumination showed normal membrane potentials, action potentials and spontaneous i.p.s.ps indistinguishable from those obtained in normal saline (Fig. 4b). A flash covering 70% of the area of the PC greatly accelerated the field potential oscillation frequency and elicited a train of i.p.s.ps in non-bursting cells, one i.p.s.p. per cycle of field potential oscillation (5 cells). Non-bursting cell spiking was eliminated during the NO-elicited train of i.p.s.ps. By contrast, activity in bursting cells was greatly increased by NO stimulation (Fig. 4c), with each burst of bursting-cell action potentials corresponding to one cycle of field potential oscillation (3 cells).

These results show that NO is involved in setting the frequency of the coherent field potential oscillation in the PC lobe of *Limax*. The field potential events in PC lobe most probably derive from postsynaptic currents elicited in non-bursting cells by bursting-cell bursts. Thus, when NO increases bursting-cell burst frequency, the field potential oscillation frequency increases. The response of non-bursting cells to NO is a change in their pattern of input from bursting cells. In the PC lobe, NO may mediate local interactions between afferent fibres from olfactory receptors, PC interneuronal neurites and pedal output dendrites²⁷ in microdomains activated by odour-elicited inputs¹⁴, analogous to intraglomerular actions of NO postulated in the olfactory bulb³.

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- Garthwaite, J., Charles, S. L. & Chess-Williams, R. *Nature* **336**, 385–388 (1988).
- Bredt, D. S. & Snyder, S. H. *Neuron* **8**, 3–11 (1992).
- Breer, H. & Shepherd, G. M. *Trends Neurosci.* **16**, 5–9 (1993).
- Bredt, D. S. et al. *Nature* **351**, 714–718 (1991).
- Croul-Ottmann, E. E. & Brunjes, P. C. *Brain Res.* **460**, 323–328 (1988).
- Davis, B. J. *J. comp. Neurol.* **314**, 493–511 (1991).
- Vincent, S. R. & Kimura, H. *Neuroscience* **46**, 755–784 (1992).
- Pape, H.-C. & Mager, R. *Neuron* **9**, 441–448 (1992).
- Moroz, L. L., Park, J.-H. & Winlow, W. *NeuroReport* **4**, 643–646 (1993).
- Chase, R. & Tolloczko, B. *J. Electron. Microsc. Techn.* **24**, 214–230 (1993).
- Ache, B. W. in *Smell and Taste in Health and Disease* (eds Getchell, T. V., Doty, R. L., Bartoshuk, L. M. & Snow, J. B.) 3–18 (Raven, New York, 1991).
- Gelperin, A. & Tank, D. W. *Nature* **345**, 437–440 (1990).
- Gelperin, A., Rhines, L., Flores, J. & Tank, D. W. *J. Neurophysiol.* **69**, 1930–1939 (1993).
- Delaney, K. R. et al. *Proc. natn. Acad. Sci. U.S.A.* **91**, 669–673 (1994).
- Gelperin, A. in *Perspectives in Neural Systems and Behavior* (eds Carew, T. & Kelley, D.) 121–136 (Liss, New York, 1989).
- Sahley, C. L. in *Connectionist Modeling and Brain Function: The Developing Interface* (eds Hanson, S. & Olson, C.) 36–73 (MIT Press, Cambridge, MA, 1990).
- Rhines, L., Sokolove, P., Flores, J., Tank, D. W. & Gelperin, A. *J. Neurophysiol.* **69**, 1940–1947 (1993).
- Cooke, I. R. C., Edwards, S. L. & Anderson, C. R. *Cell Tiss. Res.* (in the press).
- Elofsson, R., Carlberg, M., Moroz, L., Nezlín, L. & Sakharov, D. *NeuroReport* **4**, 279–282 (1993).
- Buxton, I. L. O. et al. *Circ. Res.* **72**, 387–395 (1993).
- Wink, D. A. et al. *Science* **254**, 1001–1003 (1993).
- Hrabie, J. A., Klose, J. R., Wink, D. A. & Keefer, L. K. *J. org. Chem.* **58**, 1472–1476 (1993).
- Wink, D. A. et al. *Proc. natn. Acad. Sci. U.S.A.* **90**, 9813–9817 (1993).
- Maragos, C. M. et al. *J. med. Chem.* **34**, 3242–3247 (1991).
- Carter, T. D., Bettache, N., Ogden, D. & Trentham, D. R. *J. Physiol.* **467**, 165P (1993).
- Nikol'skii, A. B., Popov, A. M. & Vasilevskii, I. V. *Soviet J. Coord. Chem.* **2**, 508–513 (1976).
- Chase, R. & Tolloczko, B. *J. comp. Neurol.* **283**, 143–152 (1989).
- Horn, R. & Marty, A. *J. gen. Physiol.* **92**, 145–159 (1988).

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Mutation in blood coagulation factor V associated with resistance to activated protein C

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ACTIVATED protein C (APC) is a serine protease with potent anticoagulant properties, which is formed in blood on the endothelium from an inactive precursor¹. During normal haemostasis, APC limits clot formation by proteolytic inactivation of factors Va and VIIIa (ref. 2). To do this efficiently the enzyme needs a non-enzymatic cofactor, protein S (ref. 3). Recently it was found that the anticoagulant response to APC (APC resistance)⁴ was very weak in the plasma of 21% of unselected consecutive patients with thrombosis⁵ and about 50% of selected patients with a personal or family history of thrombosis^{6,7}; moreover, 5% of healthy individuals show APC resistance, which is associated with a sevenfold increase in the risk for deep vein thrombosis⁵. Here we demonstrate that the phenotype of APC resistance is associated with heterozygosity or homozygosity for a single point mutation in the factor V gene (at nucleotide position 1,691, G → A substitution) which predicts the synthesis of a factor V molecule (FV Q506, or FV Leiden) that is not properly inactivated by APC. The allelic frequency of the mutation in the Dutch population is ~2% and is at least tenfold higher than that of all other known genetic risk factors for thrombosis (protein C (ref. 8), protein S (ref. 9), antithrombin¹⁰ deficiency) together.

The responsiveness of plasma to APC is measured as the ratio of two activated partial thromboplastin times, one in the presence of APC and one in its absence^{4,5,7}. This APC-sensitivity ratio (APC-SR) is normalized to the ratio obtained with a reference plasma (n-APC-SR). Resistance to APC is defined by a n-APC-SR < 0.84 (1.96 s.d. below the mean n-APC-SR in 100 healthy controls, after outlier removal).

Analysis of the parentships of 14 unrelated APC-resistant patients led to the concept of a familial form of APC resistance (or deficiency of APC cofactor II⁴) in which homozygotes and heterozygotes can be identified on the basis of the n-APC-SR (Fig. 1 legend). Further support for this came from mixing equal volumes of normal plasma and plasma from a patient classified as homozygous cofactor II-deficient (n-APC-SR, 0.38), which gave a n-APC-SR of 0.57 (Fig. 1a). This is identical to the ratio for plasma from patients heterozygous for the deficiency (mean n-APC-SR, 0.58). Mixing the plasma of four unrelated homozygous APC cofactor II-deficient patients (mean n-APC-SR, 0.40) did not alter the ratio, indicating that in all four patients the same plasma protein was missing or defective (see also refs 4 and 7).

To investigate whether APC cofactor II activity is a feature of one of the known blood coagulation proteins, APC cofactor II was assayed in a series of plasmas deficient in a single protein (Fig. 1b). All contained normal levels of APC cofactor II (60–155%) apart from plasma deficient in factor V (<5%). Addition of isolated human factor V to factor V-deficient plasma introduced factor V coagulant activity and APC cofactor II activity, suggesting that the latter is related to factor V (see also ref. 11).

Independent support for the identity of factor V with APC

cofactor II came from linkage studies in a large family with APC resistance (Fig. 2a). The human locus for the factor V gene (F5) has been mapped to chromosome 1 (1q21–25)¹². There are no reports of polymorphic F5 markers^{13–17} that can be amplified by polymerase chain reaction (PCR). Therefore, we tested the

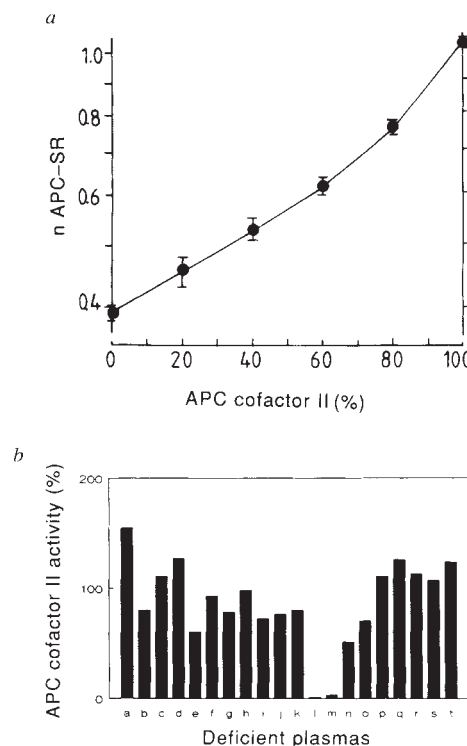


FIG. 1 Measurement of APC-cofactor II levels in plasma. a, Calibration curve for the assay of APC-cofactor II activity in plasma. APC cofactor II refers to the hypothetical new cofactor of APC⁴ which is missing or defective in individuals with APC resistance. n-APC-SRs were measured in dilutions of normal plasma (100% APC cofactor II) in plasma of a patient homozygous-deficient in APC cofactor II (0% APC cofactor II). The curve in a is the result of nine different experiments. The classification as homozygous- or heterozygous-deficient in APC cofactor II is based on the results of parentship analysis for 14 probands with APC resistance (n-APC-SR < 0.84). For 2 probands (n-APC-SR, 0.38/0.41), both parents were APC resistant (mean n-APC-SR 0.55); for 11 probands (mean n-APC-SR 0.57) one of the parents was APC resistant (mean n-APC-SR 0.59) whereas the other was not (mean n-APC-SR 0.96); for one proband (n-APC-SR 0.74), neither parent was affected (n-APC-SR 0.96/0.99). We propose that individuals can be classified as homozygotes or heterozygotes for APC cofactor II deficiency on the basis of their n-APC-SR (homozygotes: mean 0.40, $n=2$; heterozygotes: mean 0.58, range 0.51–0.67, $n=26$). b, APC cofactor II activity levels in plasmas deficient (<5%) in a single coagulation factor. Plasmas were either from patients with a congenital deficiency (a, g, f, m, g, r, s, t) or prepared by immunodepletion (b, c, d, e, j, h, i, k, l, p). Plasmas were deficient in factor II (a), factor VII (b), factor IX (c), factor X (d), factor XI (e), factor XII (j), factor XIII (g), protein C (l), protein S (i), β_2 -glycoprotein (j), antithrombin (k), factor V (l, m), factor VIII (p, q) or von Willebrand factor (r, s, t). Factor V-deficient plasma (m) was supplemented with two different concentrations, 54% (n) and 90% (o), of purified human factor V (Serbio, Gennevilliers, France), dialysed against 20 mM sodium citrate, 150 mM NaCl, 4 mM CaCl₂ and tested for APC cofactor II activity. METHODS. The APC-SR was calculated from the results of two activated partial thromboplastin times, one measured in the presence of APC and one in its absence, as before⁵. The n-APC-SR was calculated by dividing the APC-SR for the test sample by the APC-SR for pooled normal plasma. APC cofactor II activity was measured by reading the n-APC-SR for two different dilutions (1:1, 3:4) of the test plasma in APC cofactor II-deficient plasma on a calibration curve as shown in a.

segregation of microsatellite markers for several loci in the 1q21–25 region (Fig. 2b) in the family. Significantly positive results were obtained only for locus *D1S61* ($Z_{\max}$ 7.27 at $\theta=0.00$), which is located within 4 cM of the *F5* locus (see table in Fig. 2c).

We then searched for an associated mutation(s) in the factor V gene in regions containing the putative APC-binding site (corresponding to amino-acid residues 1,865–1,874)^{18,19} and the putative APC cleavage site (Arg 506)^{13,20}. Ectopic transcripts of the factor V gene from blood lymphocytes were used for first-strand synthesis of complementary DNA and subsequent amplification of the two regions coding for the APC binding and cleavage sites. Direct sequencing of the PCR fragments revealed that two patients, classified as homozygous for deficiency of APC cofactor II, were both homozygous for a guanine to adenine substitution at nucleotide 1,691 (1,691G → A) (Fig. 3a). This mutation predicts the replacement of Arg 506 (CGA) by Gln (CAA) (FV Q506 or FV Leiden). No other sequence abnormalities were detected in 225 base pairs (bp) incorporating 1,691 A or in 275 bp around the region coding for the putative APC-binding site (Fig. 3b).

If cleavage after Arg 506 is necessary for inactivation of human factor Va by APC, introduction of a glutamine at posi-

tion 506 should prevent inactivation. During coagulation, factor V is first activated by factor Xa (with formation of a 105/220K heterodimer²¹) and then processed by thrombin (with formation of a 105/74K heterodimer^{22,23}). We find that replacement of Arg 506 by Gln prevents inactivation by APC of factor Va formed after addition of factor Xa (Fig. 3c), but not that of factor Va formed after addition of α -thrombin (data not shown).

As two unrelated APC-resistant patients were homozygous for the same mutation, this alteration may predominate in other APC-resistant patients. We therefore designed a test to screen genomic DNA for the presence of the 1,691G → A substitution. The mutation is located in exon 10, 11 nucleotides 5' of the start of intron 10, and as only the first 8 nucleotides of intron 10 have been sequenced¹⁶, we generated more intron 10 sequence by heminested reverse PCR²⁴ and then designed primers for the amplification of two overlapping genomic fragments for use in genotyping.

The 267-bp fragment was digested with *Mnl*I to establish whether the allele was normal (G at 1,691) or mutated, and hybridization of the 222-bp fragment with oligonucleotides specific for each allele was used to confirm the presence of adenine at nucleotide 1,691. Using this approach, we investigated all the members of the pedigree shown in Fig. 2a. There was com-

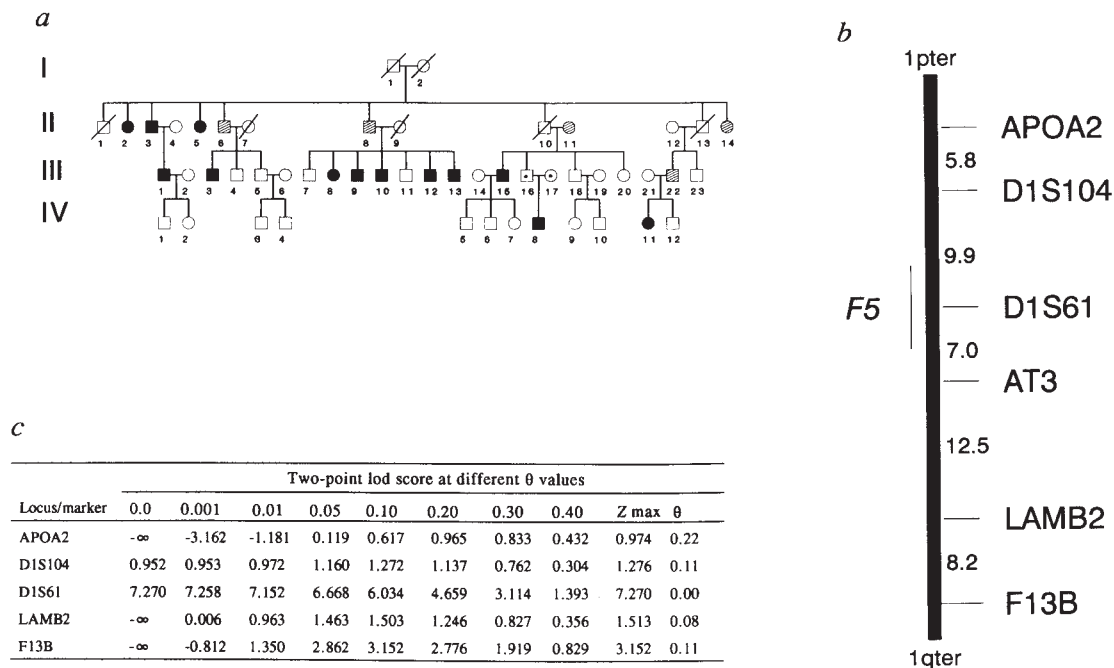


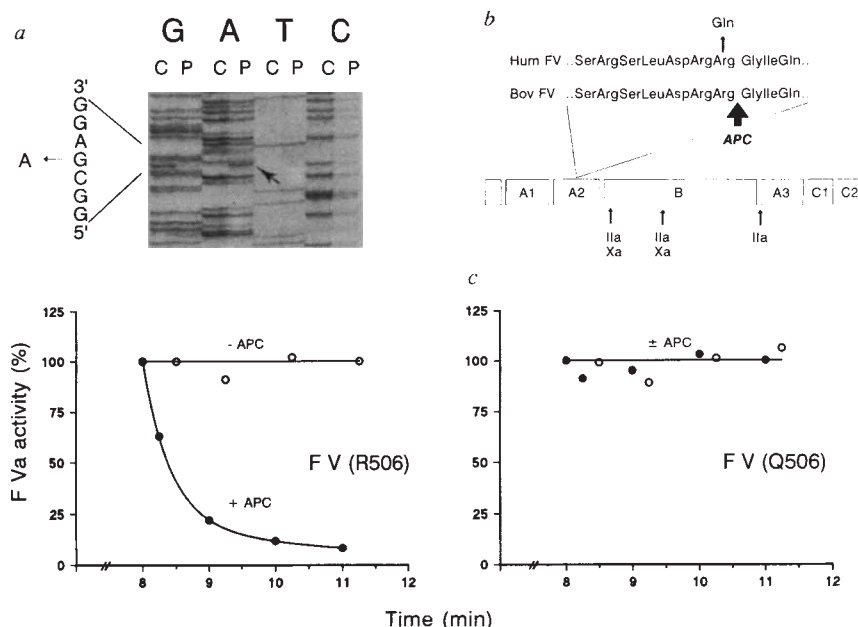
FIG. 2 Linkage analysis in a family with APC-resistance. **a**, Pedigree of a family with APC-resistance (or APC cofactor II deficiency). This pedigree forms part of a larger pedigree originally identified in our laboratory because of symptomatic type-I protein C deficiency. ●, ■, Individuals with n -APC-SR < 0.84 (mean 0.65; range 0.59–0.71, $n=13$); ○, □, individuals with n -APC-SR > 0.84 (mean 1.03; range 0.87–1.29; $n=20$); ●, ■, patients treated with oral anticoagulants (measurement of n -APC-SR in these patients is not meaningful); ○, □, individuals not tested. History of venous thrombosis: II 3, 6, 8 and 14, and III 1, 9, 20 and 22; carriers of the protein C mutation (residue at position 230, R → C): II 3, 6, 8 and 14; III 1, 5, 7, 9, 12, 18, 20, 22 and 23 and IV 1, 3, 4, 10 and 12. **b**, Integrated genetic linkage map of the q21–25 region of chromosome 1. The relative positions of the loci *APOA2*, *D1S104*, *D1S61*, *AT3*, *LAMB2* and *F13B* were derived from the NIH/CEPH Collaborative Mapping Group linkage map²⁵. The genetic distance between adjacent loci is given in cM. The *F5* locus was placed on this map within 4 cM of the *D1S61* locus by studying the segregation of markers for the *F5* and *D1S61* loci in 3 CEPH families informative for both markers (in 55 meioses, no recombination between these two loci was observed: $Z_{\max}$ 16.6 at $\theta=0.00$). **c**, Pairwise lodscores of APC-resistance with chromosome 1 markers. All available individuals of the pedigree shown in **a** were analysed. Oligonucleotide sequences for mar-

kers for the loci *ApoA2*, *D1S104*, *D1S61*, *LAMB* and *F13B* are available from the Genome Data Bank. Primers were obtained from the Dutch primer base. Three different polymorphic markers for the *AT3* locus were not informative in this family. Two-point linkage analysis was performed using the MLINK program from the LINKAGE package version 5.3 (from J. Ott). Sex-averaged lodscores are shown.

METHODS. Microsatellite markers for *ApoA2*, *D1S104*, *D1S61*, *LAMB* and *F13B* were amplified by PCR. Conditions: 50 mM NaCl, 10 mM Tris-HCl, pH 9.6, 10 mM $MgCl_2$, 0.01% BSA, 200 μ M dGTP, dATP and dTTP, 20 μ M dCTP, 0.7 μ Ci [α -³²P]dCTP, 0.43 U *Taq* polymerase (Cetus), 50 ng of each primer and 30 ng genomic DNA. 27 Cycles were run at 94 °C (1 min), 55 °C (2 min) and 72 °C (1 min), with a final elongation step of 10 min. PCR products were separated on a 6% denaturing polyacrylamide sequence gel, after which gels were dried and exposed to X-ray film. *F5* polymorphisms: A 636-bp fragment from exon 13 of the factor V gene¹⁶ was amplified by PCR using the primers 5'-TGCTGACTATGATT-ACCAGA-3' (PR-766, nucleotides 2,253–2,272; ref. 13) and 5'-GAGT-AACAGATCACTAGGAG-3' (PR-768, nucleotides 2,870–2,899; ref. 13). For PCR conditions, see legend to Fig. 4. Restriction with *Hinf*I detects a C/T dimorphism at nucleotide 2,298 (C: 0.68; T: 0.32) and a rare A/G dimorphism at nucleotide 2,411 (A: 0.98; G: 0.02). None of these markers was informative in the pedigree in **a**.

FIG. 3 Identification of the factor V gene mutation in a patient homozygous-deficient in APC cofactor II. **a**, Autoradiogram showing the nucleotide substitution in a patient classified as homozygous-deficient in APC cofactor II. Part of the nucleotide sequence of the non-coding strand of a cDNA PCR fragment (coding for amino acids 417–572 in human factor V¹³) is shown for one patient (P) and one non-APC-resistant control (C). Arrows indicate the location of the 1,691G → A transition, which predicts the replacement of Arg 506 by Gln. **b**, Schematic representation of the factor V molecule. Human factor V is a 330K glycoprotein which contains several types of internal repeats¹³. Activation by factor Xa results in the formation of a 105/220K heterodimer (A₁A₂/B'A₃C₁C₂)²²; activation by thrombin results in the formation of a 105/74K heterodimer (A₁A₂/A₃C₁C₂)²¹. APC binds to the A3 domain of factor Va^{18,19} and inhibits bovine factor Va by cleavage in the A2 domain after Arg 505 (ref. 20). The amino-acid sequences surrounding the (putative) APC cleavage site in human (Arg 506) and bovine (Arg 505) factor Va²⁶ are shown. In the APC-resistant patient, Arg 506 has been replaced by Gln. **c**, Resistance of factor Xa-activated factor V (Q506) to inactivation by APC. Al(OH)₃-adsorbed and fibrinogen-depleted plasma (for 2 h at 37 °C using 0.3 U ml⁻¹; Arvin) containing either factor V R506 or factor V Q506 was treated with factor Xa (2 nM) in the presence of 20 mM CaCl₂ and 20 μM PS/PC (25/75). After 8 min, when the factor Va level had reached a plateau, 1.9 nM APC or buffer was added. At different time intervals, 10 μl sample was diluted 1/100 in 'stop' buffer (50 mM Tris-HCl, pH 7.9, 180 mM NaCl, 0.5 mg ml⁻¹ OVA, 5 mM CaCl₂ and 0.5 μg ml⁻¹ heparin) and directly assayed for factor Va activity as described²⁷. The factor Va activity measured after complete activation of 0.70 U ml⁻¹ FV (R506) (0.64 μM thrombin min⁻¹) or 0.49 U ml⁻¹ FV (Q506) (0.20 μM thrombin min⁻¹) is arbitrarily put at 100%; ○, no APC; ●, +APC.

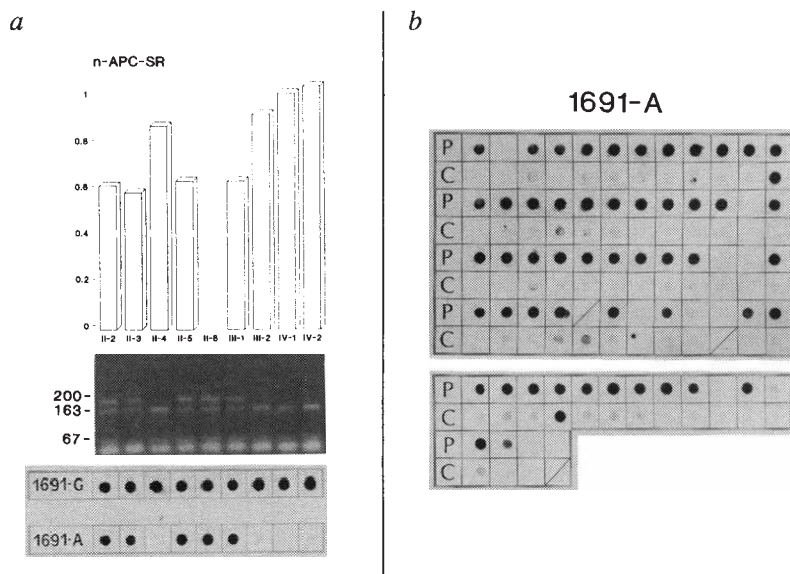
METHODS. cDNA synthesis: RNA was isolated²⁸ from the lymphocyte fraction of 10 ml citrated blood of consenting patients and non-APC-resistant controls. RNA (1 μg) was used as template for first-strand cDNA synthesis in the presence of mixed random hexamers using the



superscript kit (BRL). Amplification of cDNA fragments: the primers 5'-GCATTTACCTCATGGAGTG-3' (PR-764, nucleotides (nt) 1,421–1,440; ref. 13) and 5'-CAAGAGTAGTTATGCTCTCAGGCAC-3' (PR-856, nt 1,867–1,891; ref. 13) amplify the region coding for residues 417–572 which contains the putative APC cleavage site; the primers 5'-CACGTGGTTCACCTTTCACGG-3' (PR-849, nt 5,608–5,627; ref. 13) and 5'-TGTGGTATAGCAGGACTTCAGGTA-3' (PR-848, nt 6,040–6,063; ref. 13) amplify the region coding for amino-acid residues 1,812–1,963, which contains the APC-binding region. PCR conditions are described in Fig. 4 legend. PCR fragments were purified on ultra-low-gelling temperature agarose and directly sequenced as before²⁹ using the same primers as in the PCR reaction. One additional primer was synthesized to aid sequencing of the APC-binding region: 5'-TATAAGATCCACCATTGT-3' (PR-847, nt, 5,905–5,927; ref. 13).

FIG. 4 Association of APC resistance with the presence of a 1,691A allele of factor V. **a**, Cosegregation of 1,691A with APC resistance. Upper, position of the individuals in the pedigree shown in Fig. 2a and their n-APC-SR, if available (II6 was on oral anticoagulant treatment). Middle, *Mn*II digestion of the 267-bp PCR fragment. Lower, dot-blot hybridization of the 222-bp fragment with the biotinylated oligonucleotide specific for the 1,691A allele (PR-1005). **b**, Dot-blot hybridization of the 222-bp PCR fragments of 64 thrombosis patients with n-APC-SR < 0.84 and of their 64 matched controls with the biotinylated oligonucleotide specific for the 1,691A allele (PR-1005). All patients (P) and controls (C) gave their informed consent. Slashes denote positions of failed PCR reactions in this experiment.

METHODS. Amplification of genomic fragments containing 1,691G/A. For *Mn*II digestion a 267-bp fragment was amplified using as 5' primer 5'-TGCCAGTGCTTACAAG-ACCA-3' (PR-6967; nt 1,581–1,602; ref. 13) and as 3' primer 5'-TGTTATCACACTGGTGCTAA-3' (PR-990; nt 127 to –146 in intron 10). For dot-blot hybridization, a 222-bp fragment was amplified using as 5' primer 5'-GAGAGAC-ATCGCCTCTGGGCTA-3' (PR-6966, nt 1,626–1,647; ref. 13) and as 3' primer PR-990. Conditions: 125 μl of a mixture containing 54 mM Tris-HCl, pH 8.8, 5.4 mM MgCl₂, 5.4 μM EDTA, 13.3 mM (NH₄)₂SO₄, 8% DMSO, 8 mM β-mercaptoethanol, 0.4 mg ml⁻¹ BSA, 0.8 mM of each nucleoside triphosphate, 400 ng of each primer, 200–500 ng DNA and 2 U *Taq* polymerase were subjected to 36 cycles of 91 °C (40 s), 55 °C (40 s) and 71 °C (2 min). The 267-bp fragment (7–10 μl) was digested with 0.4 U *Mn*II (Biolabs): the 1,691G fragment will give fragments of 67, 37 and 163 bp, whereas the 1,691A fragment will give fragments of 67 and 200 bp. The 222-bp fragment (~100 ng) was used for dot-blot



hybridization with biotinylated sequence-specific oligonucleotides (5'-TGGACAGGCgAGGAATAC-3' (PR-1006; nt 1,682–1,699; ref. 13) for detection of 1,691G and 5'-TGGACAGGCgAGGAATAC-3' (PR 1005) for detection of 1,691A. Procedures have been described³⁰. After hybridization, stringency washing with PR-1006 was at 53 °C, and with PR-1005 at 52 °C.

plete cosegregation of heterozygosity for the 1,691G → A mutation with APC resistance (n-APC-SR < 0.84) as shown for part of the pedigree (Fig. 4a). Four patients (II.6, II.8, II.14, III.22), for whom no n-APC-SR could be determined because of oral anticoagulant treatment, were found to be heterozygous.

In a previous study of 301 consecutive patients who had suffered a first episode of deep vein thrombosis and of 301 age- and sex-matched controls from the general population, 64 APC-resistant thrombosis patients had been identified⁵. These 64 patients and their 64 controls were screened for the presence of the G → A substitution. Seventy had n-APC-SR < 0.84 (64 patients, 6 controls), of which 56 carried the mutation (53 patients, 3 controls), in both alleles in six of the patients (mean n-APC-SR, 0.43; range, 0.41–0.44) and in one allele in 50 patients (mean n-APC-SR, 0.57; range, 0.50–0.67). The remaining 14 APC-resistant individuals did not carry the mutation and had only a marginally reduced n-APC-SR (mean n-APC-SR, 0.78; range, 0.70–0.83). None of the 58 individuals who were not APC-resistant carried the mutation (mean n-APC-SR, 0.99; range, 0.83–1.19). Further, none of 100 consecutive thrombosis patients with n-APC-SR > 0.84 was a carrier of the mutation, whereas 3 of their 100 matched controls were, as expected. These three (n-APC-SR values 0.57, 0.58 and 0.59) were the only controls with n-APC-SR < 0.84.

Our results show that 80% of the individuals with n-APC-SR < 0.84 and 100% of those with n-APC-SR < 0.70 are heterozygotes or homozygotes for the mutation and that all carriers of the mutation have n-APC-SR < 0.7. The high frequency of the mutated allele in the Dutch population (about 2%) combined with our previous finding⁵ that APC resistance is a common and strong risk factor for deep vein thrombosis, makes this hereditary factor V defect the most common hereditary blood coagulation disorder identified so far. A founder effect may be involved in the spread of this disorder in the population, as suggested by the overrepresentation of the common *HinfI* allele of the factor V gene (cytosine at nucleotide 2,298; Fig. 2 legend) in carriers of the factor V Leiden mutation; the frequency of 2,298C was 0.96 in 53 carriers of the mutation and 0.73 in 69 non-carriers ($\chi^2_{diff} = 30.4$, d.f. = 1; $P < 0.001$). □

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- Esmon, C. T. *Arteriosclerosis and Thrombosis* **12**, 135–145 (1992).
- Walker, F. J. & Fay, P. J. *FASEB J.* **6**, 2561–2567 (1992).
- Walker, F. J. *J. biol. Chem.* **265**, 5521–5524 (1990).
- Dahlbäck, B., Carlsson, M. & Svensson, P. J. *Proc. natn. Acad. Sci. U.S.A.* **90**, 1004–1008 (1993).
- Koster, T. et al. *Lancet* **342**, 1503–1506 (1993).
- Svensson, P. J. & Dahlbäck, B. *New Engl. J. Med.* **300**, 517–522 (1994).
- Griffin, J. H., Evatt, B., Wideman, C. & Fernandez, J. A. *Blood* **82**, 1989–1993 (1993).
- Allaart, C. F. et al. *Lancet* **341**, 134–138 (1993).
- Engesser, L., Broekmans, A. W., Briët, E., Brommer, E. J. P. & Bertina, R. M. *Ann. int. Med.* **106**, 677–682 (1987).
- Hirsh, J., Piovella, F. & Pini, M. *Am. J. Med.* **87**, 345–385 (1989).
- Dahlbäck, B. & Hildebrand, B. *Proc. natn. Acad. Sci. U.S.A.* **91**, 1396–1400 (1994).
- Wang, H., Riddell, D. C., Quinto, E. R., MacGillivray, R. T. A. & Hamerton, J. L. *Genomics* **2**, 324–328 (1988).
- Jenny, R. J. et al. *Proc. natn. Acad. Sci. U.S.A.* **84**, 4846–4850 (1987).
- Kane, W. H. & Davie, E. W. *Proc. natn. Acad. Sci. U.S.A.* **83**, 6800–6804 (1986).
- Kane, W. H., Ichinose, A., Hagen, F. S. & Davie, E. W. *Biochemistry* **26**, 6508–6514 (1987).
- Cripe, L. D., Moor, K. D. & Kane, W. H. *Biochemistry* **31**, 3777–3785 (1992).
- Shen, N. L. L. et al. *J. Immunol.* **150**, 2992–3001 (1993).
- Walker, F. J., Scandella, D. & Fay, P. J. *J. biol. Chem.* **265**, 1484–1489 (1990).
- Walker, F. J. & Fay, P. J. *J. biol. Chem.* **265**, 1834–1836 (1990).
- Odegard, B. & Mann, K. J. *J. biol. Chem.* **262**, 11233–11238 (1987).
- Suzuki, K., Dahlbäck, B. & Stenflo, J. J. *J. biol. Chem.* **257**, 6556–6564 (1982).
- Monkovic, D. D. & Tracey, P. B. *Biochemistry* **29**, 1118–1128 (1990).
- Yang, X. J. et al. *Biochem. J.* **272**, 399–406 (1990).
- Triglia, T., Peterson, M. G. & Kemp, D. J. *Nucleic Acids Res.* **16**, 8186 (1988).
- NIH/CEPH Collaborative Mapping Group. *Science* **258**, 67–86 (1992).
- Quinto, E. R., Esmon, C. T., Mann, K. G. & MacGillivray, R. T. A. *J. biol. Chem.* **267**, 2971–2978 (1992).
- Pieters, J. & Lindhout, T. *Blood* **72**, 2048–2052 (1988).
- Chromczynski, P. & Sacchi, N. *Analyt. Biochem.* **162**, 156–159 (1987).
- Reitsma, P. H., Poort, S. R., Allaart, C. F., Briët, E. & Bertina, R. M. *Blood* **78**, 890–984 (1991).
- Verduyn, W. et al. *Hum. Immunol.* **37**, 59–67 (1993).

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CTL induction by a tumour-associated antigen octapeptide derived from a murine lung carcinoma

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MANY mouse and human tumours express major histocompatibility complex (MHC) class I-associated antigens that constitute targets for syngeneic cytotoxic T lymphocytes (CTL). Genes encoding such antigens were isolated from a mouse mastocytoma and from human melanomas by genetic methods^{1,2}. Isolation and characterization of MHC class I-associated peptides has enabled specific anchor residues to be identified that are typical of peptides that bind to distinct class I molecules³. Moreover, CTL specific to particular MHC-peptide combinations have been used to identify naturally occurring antigenic peptides in cell extracts and enabled them to be sequenced directly^{4–6}. Most known MHC ligands are of viral origin or are self peptides derived from normal proteins⁷. Here we use total acid extraction and repeated fractionation to isolate and sequence Lewis lung carcinoma (3LL)-specific peptide(s), which shows sequence homology to the connexin 37 protein. Synthetic octamers based on these sequences bind to 'empty' H-2K^b molecules on RMA-S cells, sensitize RMA-S cells to lysis by specific anti-3LL CTL, and induce anti-tumour CTL. The tumour-associated peptide originates from mutated connexin 37 expressed in 3LL.

The high metastatic clone D122 of the 3LL carcinoma is characterized by low cell-surface expression of H-2K^b molecules and poor immunogenicity. Transfection of H-2K^b converts D122 to a non-metastatic and highly immunogenic phenotype, whereas H-2D^b, expressed on D122 cells, does not serve as a restriction element for anti-tumour CTL^{8–10}. In preliminary experiments, the H-2K^b-D122 transfectant K^b39.5 was used for isolating K^b-bound peptides^{3,5}. Sensitization of H-2 'empty' RMA-S cells, by the high-pressure liquid chromatography (HPLC)-fractionated peptides, to lysis by anti-K^b39.5 CTL repeatedly revealed the presence of prominent active peptide fractions (not shown), but fractions from 2×10^{10} tissue cultured cells yielded insufficient quantity for sequence analysis by mass spectroscopy. We therefore injected large groups of nude mice with K^b39.5 tumour cells. Total acid extraction rather than isolation of K^b-bound peptide complexes was used to increase the amount of extractable peptides. The total acid peptide fraction ($M_r \leq 5,000$) was tested for the presence of K^b-binding peptides and anti-tumour properties.

Figure 1a shows stabilization of K^b molecules on RMA-S, H-2 negative cells, incubated with total peptides. D^b expression was also induced by the same fraction (not shown). Immunization of syngeneic C57BL/6 mice with peptide-loaded RMA-S cells produced a tumour-specific CTL response, comparable to that obtained after immunization with K^b39.5 tumour cells (Fig. 1b). It also retarded tumour growth and metastasis in C57BL/6 mice bearing 18-day-old D122 tumours (Fig. 1c, d). Thus, the crude peptide fraction contained peptides presentable by syngeneic RMA-S cells that conferred anti-tumour activity.

Crude peptide fractions were separated by reversed-phase HPLC (Fig. 2) and individual fractions were tested, in six independent assays, for lysis by anti-K^b39.5 CTL (Fig. 2a). The most active fractions (31,32) were pooled, rechromatographed

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and tested as before (Fig. 2b). Active fractions (41,42) were pooled and separated again (Fig. 2c). Active peak 30 (100 pmol) was sequenced by Edman degradation. The first seven residues of a major peptide, Phe-Glu-Gln-Asn-Thr-Ala-Gln, were unambiguously determined. The eighth residue was questionably Ala or Pro, and a possible ninth residue, glycine, could also be determined. An NBRF data bank search revealed homology with a peptide from the mouse gap-junction protein connexin 37 (Phe-Glu-Cys-Asn-Thr-Ala-Gln-Pro-Gly, positions 52–60). Because most known K^b-restricted peptides, are eight amino acids long⁷, octameric synthetic peptides were prepared representing connexin 37 positions 52–59, and two variants of the deduced

sequences with Pro (MUT 1) or Ala (MUT 2) at position eight (Fig. 2d). Figure 3a shows that MUT 1, MUT 2 and the prototype K^b-restricted peptide VSV 52–59 all stabilized K^b expression on RMA-S cells, whereas connexin 37 had no effect.

Similar testing with anti-D^b antibodies revealed no binding (Fig. 3a). Both MUT 1 and MUT 2, but not connexin 37 or VSV peptides, were active in cytotoxic assays with anti-K^b39.5 CTL (Fig. 3b, c). Lytic activity could be blocked by preincubation of peptide-loaded RMA-S cells with anti-K^b antibody 20-8-4 but not with anti-D^b antibody 28-14-8 (Fig. 3b). Sensitization for lysis by synthetic peptides showed maximal effect at 100 nM for MUT 2 and MUT 1, but lysis was detected even after sensi-

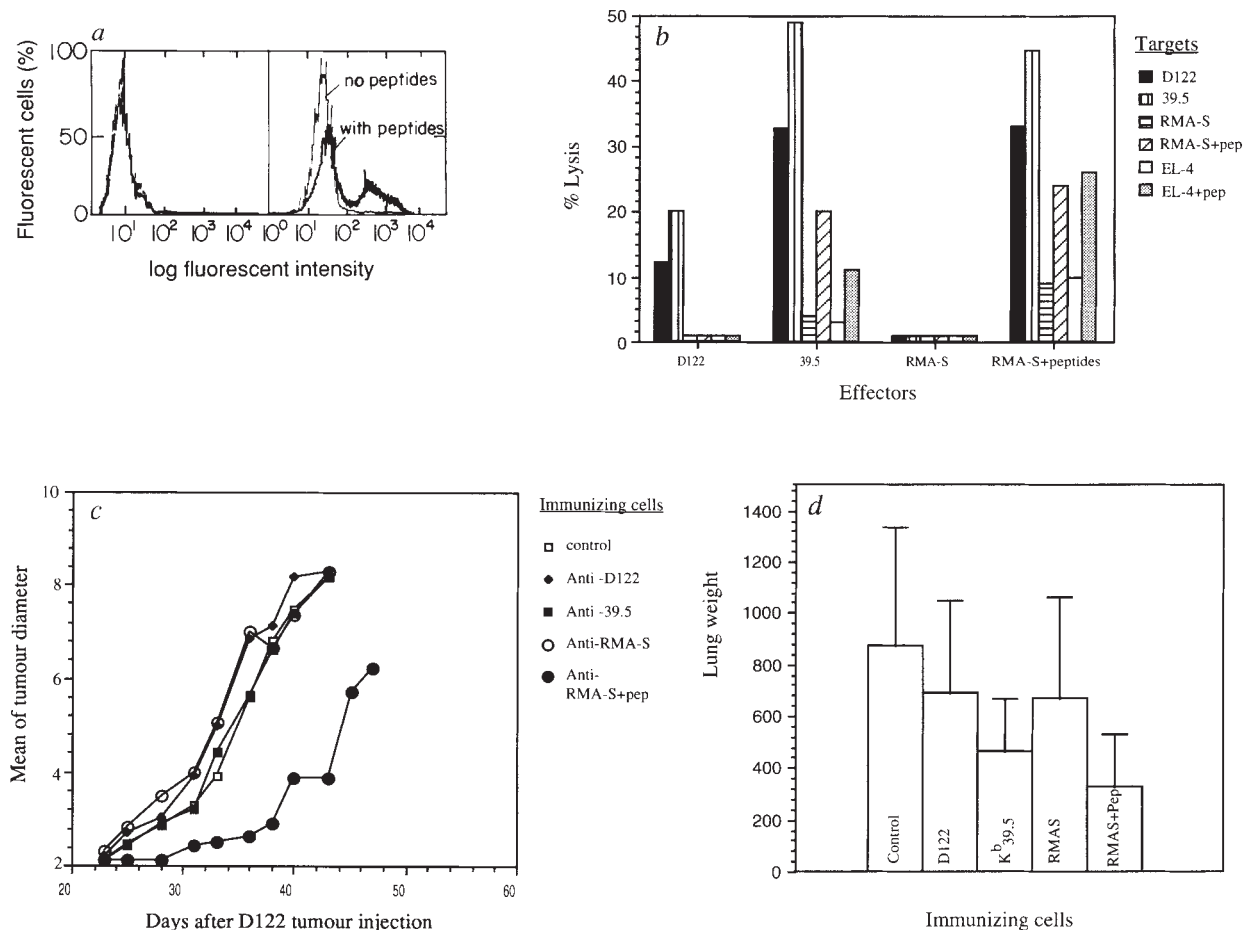


FIG. 1 H-2K^b stabilization and immunogenicity of total acid-extracted peptides. *a*, Left, anti-K^b staining of RMA-S cells²² without peptides; right, anti-K^b staining of RMA-S cells loaded with total peptide extract. *b*, *In vitro* lytic activity of CTL induced by peptide-bound RMA-S cells. *c*, *d*, Mean tumour diameters (in mm) and lung weights (in mg) of D122 tumour-bearing mice vaccinated with peptide-loaded RMA-S cells. *P* < 0.0001 between the RMA-S+ peptide vaccinated group and all other groups in *c*, and *P* values are 0.028 and 0.0004 for groups vaccinated with K^b 39.5 and peptide-loaded RMA-S cells, respectively, as compared to the non-vaccinated group (*d*).

METHODS. Total acid-extracted peptides were prepared from K^b39.5 cells grown as subcutaneous tumours in 150 CD1 nude mice. Non-necrotic (1–2 cm) tumours were homogenized in PBS containing 0.5% NP-40, 10 µg ml⁻¹ soybean trypsin inhibitor, 5 µg ml⁻¹ leupeptin, 8 µg ml⁻¹ aprotinin, 0.5 mM PMSF, stirred for 30 min at 4 °C, titrated with 10% TFA to final concentration of 0.1% TFA and further processed²³. One-third of the peptidic fraction (*M*_r < 5,000) was dissolved in Iscove's modified Dulbecco's medium (IMDM) containing combined antibiotics and 5 × 10⁻⁵ M β-mercaptoethanol (βME), and 2/3 of the material was dissolved in 0.1% TFA for HPLC separation. For peptide loading, RMA-S cells were precultured, 36–48 h at 26 °C in RPMI medium supplemented with 10% FCS, 1 mM glutamine, 1 mM

non-essential amino acids, 1 mM sodium pyruvate, combined antibiotics, 10 mM HEPES, pH 7.4, 2 × 10⁻⁵ M βME (RPMI-HEPES medium). Cells were irradiated (5,000 rads) for immunization *in vivo*, labelled with ³⁵S-methionine for target preparation, or left untreated and then incubated in a small volume (50–100 µl) containing 5 × 10⁴–8 × 10⁶ cells) with an equal volume of 250 µg ml⁻¹ total extracted peptides, for 30 min at 26 °C and 3.5 h at 37 °C. For flow cytometry, washed cells were stained with anti-K^b antibody 20-8-4. For CTL assays, effector lymphocytes were derived from C57BL/6 mice that had been immunized with 2 × 10⁶ irradiated (5,000 rads) D122, K^b39.5, RMA-S and peptide-loaded RMA-S cells⁹. Splenocytes were restimulated on irradiated (5,000 rads) and mytomycin C-treated (80 µg ml⁻¹ per 10⁶ cells) K^b39.5 cells for 4 days, and reacted with 5 × 10³ ³⁵S-methionine-labelled target cells, at an effector-to-target ratio of 50:1, for 5 h at 37 °C (ref. 9). Spontaneous release did not exceed 30% of maximal release. The standard error was less than 5% of the means of the wells. For *in vivo* vaccination assays, C57BL/6 mice were inoculated into the footpad with 2 × 10⁵ D122 cells per mouse. Eighteen days later, groups of mice were immunized i.p. five times at 7-day intervals with inactivated (5,000 rads) parental D122 cells, K^b39.5, RMA-S cells and peptide-loaded RMA-S cells. Controls were non-immunized mice. Tumour growth and metastasis were determined as described⁹.

zation with peptides at 5 nM (Fig. 3d). These synthetic peptides also induced anti-tumour CTL *in vivo*. Splenocytes from mice immunized with RMA-S cells loaded with synthetic peptides exhibited similar specificities as anti-K^b39.5 CTL (Fig. 3e). Anti-MUT 1/2 and anti-K^b39.5 CTLs also effectively lysed another spontaneous C57BL/6 lung carcinoma, CMT 64 (ref. 11), but not other tumours of C57BL/6 origin like sarcomas MCA 102 and 105, T-cell lymphoma EL4, melanoma B16 or the normal fibroblast cell-line BLK.CL4 (not shown).

Operationally, both MUT 1 and MUT 2 can be defined as K^b-restricted tumour-associated antigen peptides, but their sequences do not conform with the consensus allele-specific motifs for K^b, which specify Tyr or Phe in position 5, Leu or Ile in position 8 as anchor positions, and occasionally, Tyr in position 3 as an auxiliary anchor^{3,5}. Models delineating the complexes of K^b and each of the three peptides (MUT 2, MUT 1 and connexin 37) were built, based on the structures of K^b-VSV and K^b-SEV, determined by X-ray crystallography (data not shown)^{12,13}. Replacement of VSV side chains according to MUT 1, MUT 2, and connexin 37 sequences showed that all three peptides could be accommodated by the K^b binding groove. The interactions

of the N and C termini and backbone atoms of the peptide with the site are preserved. The interaction of VSV-Tyr/P5 with pocket C are successfully imitated by MUT 1/2-Glu/P2 and Thr/P5, and the interactions of VSV-Tyr/P3 with pocket D can be replaced by MUT 1/2-Gln/P3, but not by Cys/P3 in the connexin 37 peptide.

The energies of the three K^b peptides and the K^b-VSV complexes were minimized using the ENCAD program¹⁴. The interaction energies between VSV, MUT 2, MUT 1 and K^b are within 3 kcal mol⁻¹ of one another, -133, -131 and -134 kcal mol⁻¹, respectively, whereas the interaction energy between connexin 37 and K^b is -122 kcal mol⁻¹. This analysis indicates that MUT 2 and MUT 1, but not the connexin 37 peptide, could bind stably to K^b molecules. The difference between MUT 1 (Pro) and MUT 2 (Ala) in the eighth position only marginally influences CTL recognition as this amino acid is anchored in pocket F and not exposed to CTL binding.

To examine whether MUT 1 and/or MUT 2 are actually derived from a mutated connexin 37 gene, reverse transcription-polymerase chain reactions (RT-PCR) were done on RNAs isolated from D122, K^b39.5, or normal C57BL/6 lungs using a 3'

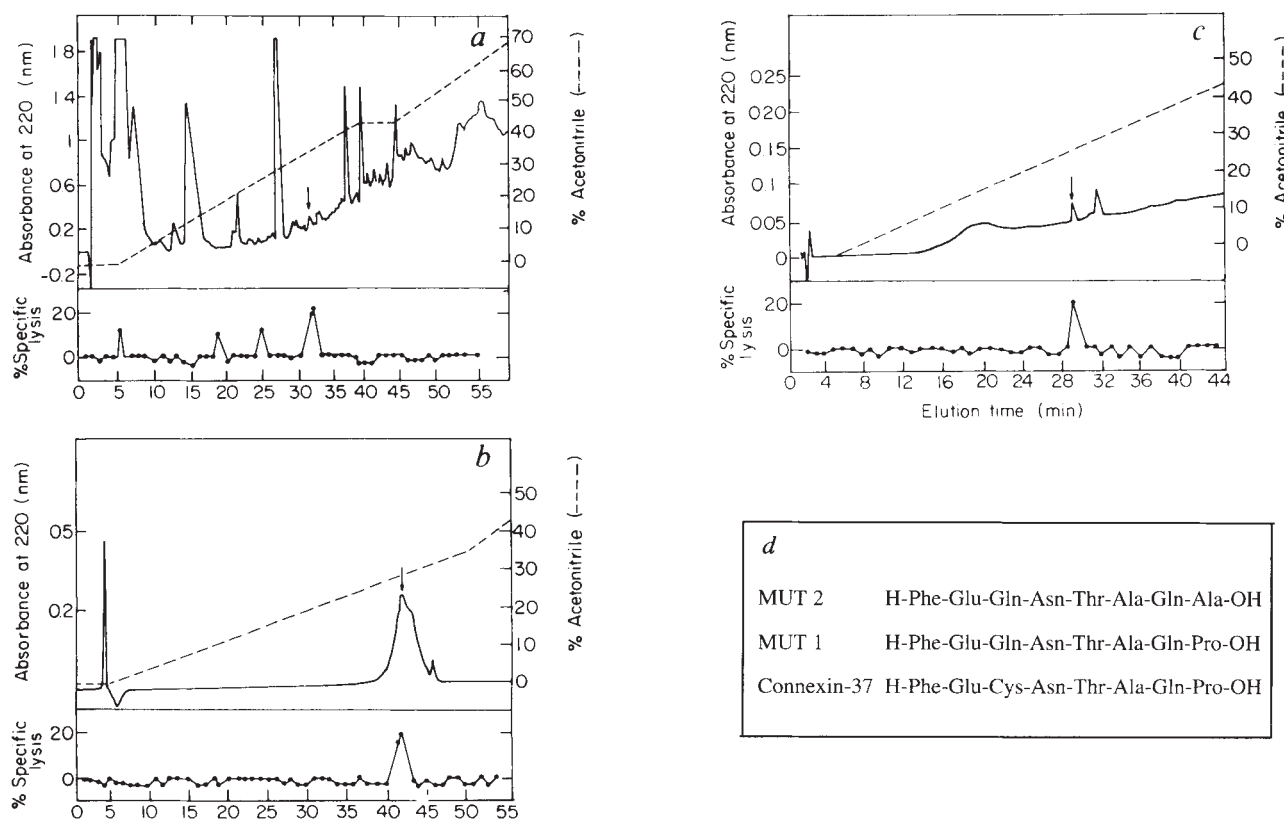


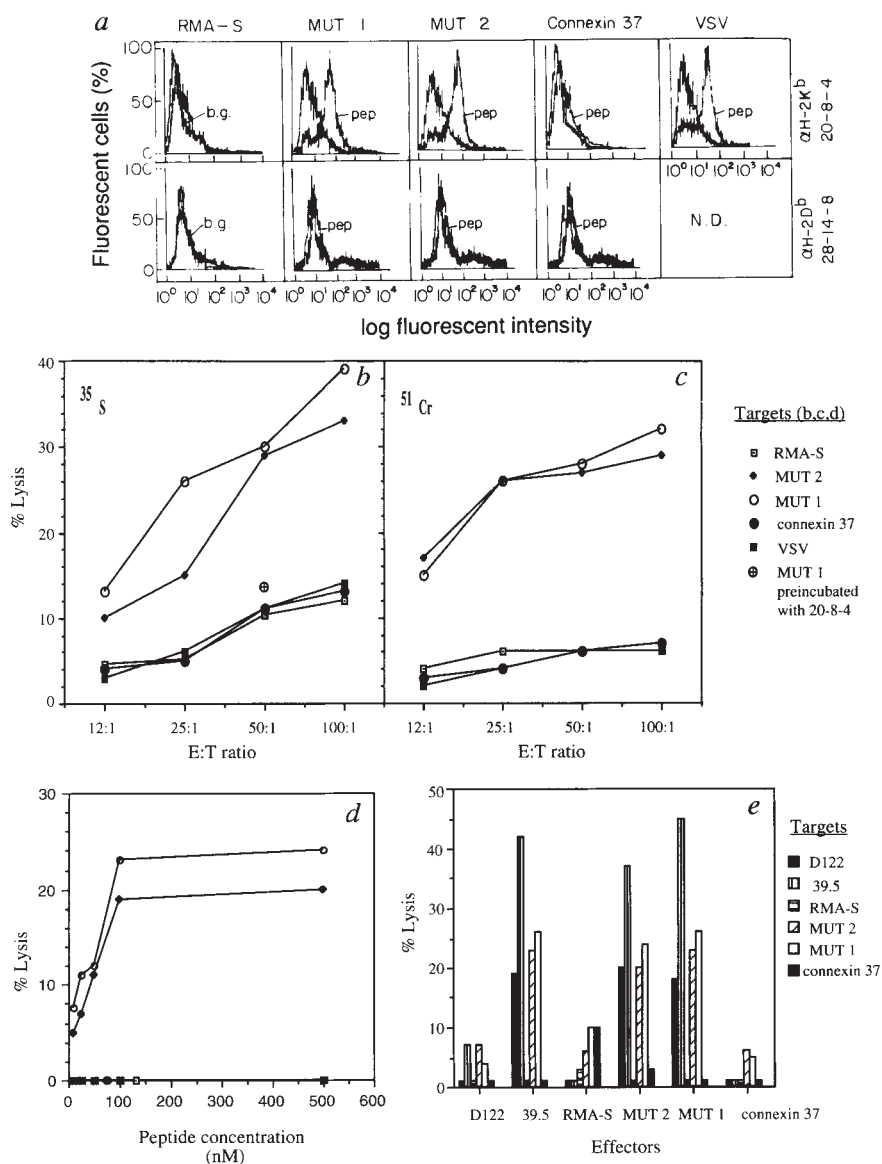
FIG. 2 Reversed-phase HPLC separation of total acid-extracted peptides and detection of naturally occurring CTL epitopes. Separations used a Merck Hibar column (250 × 4 mm) repacked with Lichosorb RP-8 (5 µm) (Merck). Eluents: solution A, 0.1% TFA in water (v/v); solution B, 0.1% TFA in 70% acetonitrile in water (v/v). The gradients are described in the figures (a–c, upper panels). a, HPLC profile (upper) and CTL activity (lower) of the first separation (one of eight). Fractions of 1 ml, at a flow rate of 1 ml min⁻¹ were collected. Fractions 31 and 32 (arrow) were pooled from all separations. b, Second HPLC separation: pooled fractions from a, concentrated to 1 ml, were rechromatographed. Active fractions 41 and 42 (arrow) were collected for further processing. c, Third HPLC separation. Active fraction 30 (arrow) was sequenced. d, Amino-acid sequence of MUT 2, MUT 1 and connexin 37 (52–59). Sequencing was by Edman degradation on an Applied Biosystem protein sequencer model 475A with an on-line 120A PTH analyser. Synthetic

peptides were prepared by standard Merrifield solid-phase methods, using t-boc chemistry on an applied biosystem 430A, and purified by HPLC.

METHODS. Individual fractions from two of eight separations were freed of acetonitrile and TFA by repeated drying in a vacuum centrifuge. Neutral fractions were resuspended in 0.6 ml RPMI-HEPES medium. RMA-S cells were incubated at 26 °C for 48 h, labelled with ³⁵S-methionine for 8 h, and 5 × 10⁵ cells in 50 µl RPMI medium were incubated with 50 µl of peptides from each fraction, in duplicates, at 26 °C for 30 min. Restimulated lymphocytes from K^b39.5 immunized mice were added at a 100:1 ratio. After 5 h at 37 °C, plates were centrifuged and processed⁹. Per cent specific lysis was calculated as before⁹. RMA-S cells without peptides were used as a negative control and K^b39.5 cells were used as a positive control. The figures present lysis of individual fractions minus lysis of RMA-S cells (3–5%).

FIG. 3 Stabilization of K^b expression and lysis of RMA-S cells as mediated by MUT 1 and MUT 2 peptides. **a**, H-2K^b and H-2D^b cell surface expression of RMA-S cells incubated with 5 μ M synthetic peptides, MUT 2, MUT 1, connexin 37 (amino acids 52–59), or VSV (amino acids 52–59). RMA-S and peptide-loaded cells were stained with monoclonal anti-K^b antibody 20-8-4 (upper), and anti-D^b antibody 28-14-8 (lower). Left, RMA-S cells stained with FITC-conjugated goat-anti mouse immunoglobulin (background) or K^b/D^b and second antibody. Other panels: peptide-loaded RMA-S cells stained with anti K^b/D^b compared to anti-K^b/D^b-stained RMA-S cells. Backgrounds of peptide-loaded RMA-S cells overlap with the background of RMA-S cells in the left panels and are not shown in other panels. **b**, **c**, *In vitro* lytic activity of CTL induced by K^b39.5 cells, tested on RMA-S cells loaded with 5 μ M of various synthetic peptides and inhibition of lysis by anti-K^b antibody. RMA-S cells were labelled as described for Fig. 1. Inhibition of lysis of MUT 1-loaded RMA-S cells was done in the presence of 1:40 dilution of 20-8-4 ascitic fluid (in **b**). Cells were also labelled with ⁵¹Cr by incubation of 10×10^6 RMA-S cells in 100 μ l IMDM medium supplemented with 100 μ Ci of ⁵¹Cr-Na₂CrO₄ for 90 min at 31 °C (in **c**). Cells were washed and used as targets in 5-h cytotoxicity assays as in Fig. 1. The data represent an average of four independent assays for each form of cell labelling. **d**, Dose-response: 5–500 nM of the various peptides were incubated with ³⁵S-methionine-labelled RMA-S cells, and reacted with CTL for 5 h as described in Fig. 1. Lysis at effector:target (E:T) ratio of 50:1 is shown. **e**, *In vitro* lytic activity of CTL induced by MUT 1 and MUT 2 RMA-S-loaded cells. E:T ratio, 25:1. Experimental details were described in Fig. 1.

METHODS. For inhibition of lysis by antibodies, peptide-loaded and labelled RMA-S cells were incubated with 1:40, 1:80, and 1:160 dilutions of anti-K^b 20-8-4, or anti-D^b 28-14-8 for 1 h at room temperature. Effector cells were added at E:T ratio of 50:1 for 5 h as described in Fig. 1. The data presented are from a single 20-8-4 concentration (1:40), with MUT 1-RMA-S as target and K^b39.5-induced CTL as effectors. Similar inhibition was observed with CTL induced by MUT 1-RMA-S loaded cells incubated with



MUT 1-RMA-S or K^b39.5 cells as targets. Anti-D^b 28-14-8 did not inhibit lysis (data not shown).

primer derived from the connexin 37 sequence (501–518) and variant 5' primers overlapping the putative mutation sites at their 3' end (Fig. 4a) as described in ref. 15. Priming with the TGT(Cys)-containing primer amplified products of ~300 bp from all complementary DNAs (Fig. 4b, primer 1). In contrast, the mutated CAG primer, which represents a Gln sequence, amplified similar products only in tumour cells (Fig. 4b, primer 2). Direct sequencing confirmed that connexin 37 sequences were amplified, indicating that Gln-mutated, as well as normal connexin 37 are expressed in the tumour (Fig. 4c). The eighth amino acid of the peptide was found to be Pro (CCG) and not Ala (GCN) (Fig. 4c). Connexin 37 is abundant in lungs and belongs to a family of transmembrane proteins that form intercellular hydrophilic channels called gap junctions^{16,17}. Formation of gap junctions is dependent on a small number of cysteine residues in the extracellular domains of connexins, among them Cys 54, which is located on the first extracellular domain of connexin 37 and is replaced by glutamine in the lung carcinoma peptides. Other connexins, such as the human connexins 26 and 43, were suggested to act as tumour-suppressor genes in breast and are downregulated in human breast carcinomas^{18,19}. Whether mutations in connexin 37 contribute to the malignancy of lung carcin-

omas or represent sporadic events during tumour progression is not clear. But transfection of connexin 43 caused growth retardation of glioma cells *in vitro* and transfection of connexin 32 caused growth retardation of hepatoma cells *in vivo*, indicating negative correlation between intercellular communication and tumorigenicity^{20,21}.

Thus, we have isolated a 3LL tumour peptide, derived from a mutated connexin 37 gene, whose synthetic homologues bind to H-2K^b and sensitize RMA-S cells to lysis by anti-3LL CTL. These peptides induce CTL responses against 3LL-derived clones and against an independent C57BL/6 lung carcinoma, suggesting that common lung carcinoma tumour-associated antigens may exist. Recent immunotherapy experiments indicate that peptide vaccination may constitute an effective antimetastatic modality (manuscript in preparation). □

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- Van Den Eynde, B., Lethe, B., Van Pel, A., De Plaen, E. & Boon, T. J. *exp. Med.* **173**, 1373–1384 (1991).
- Van Der Bruggen, P. et al. *Science* **254**, 1643–1647 (1991).
- Falk, K., Rotzschke, O., Stevanovic, S., Jung, G. & Rammensee, H. G. *Nature* **351**, 290–296 (1991).

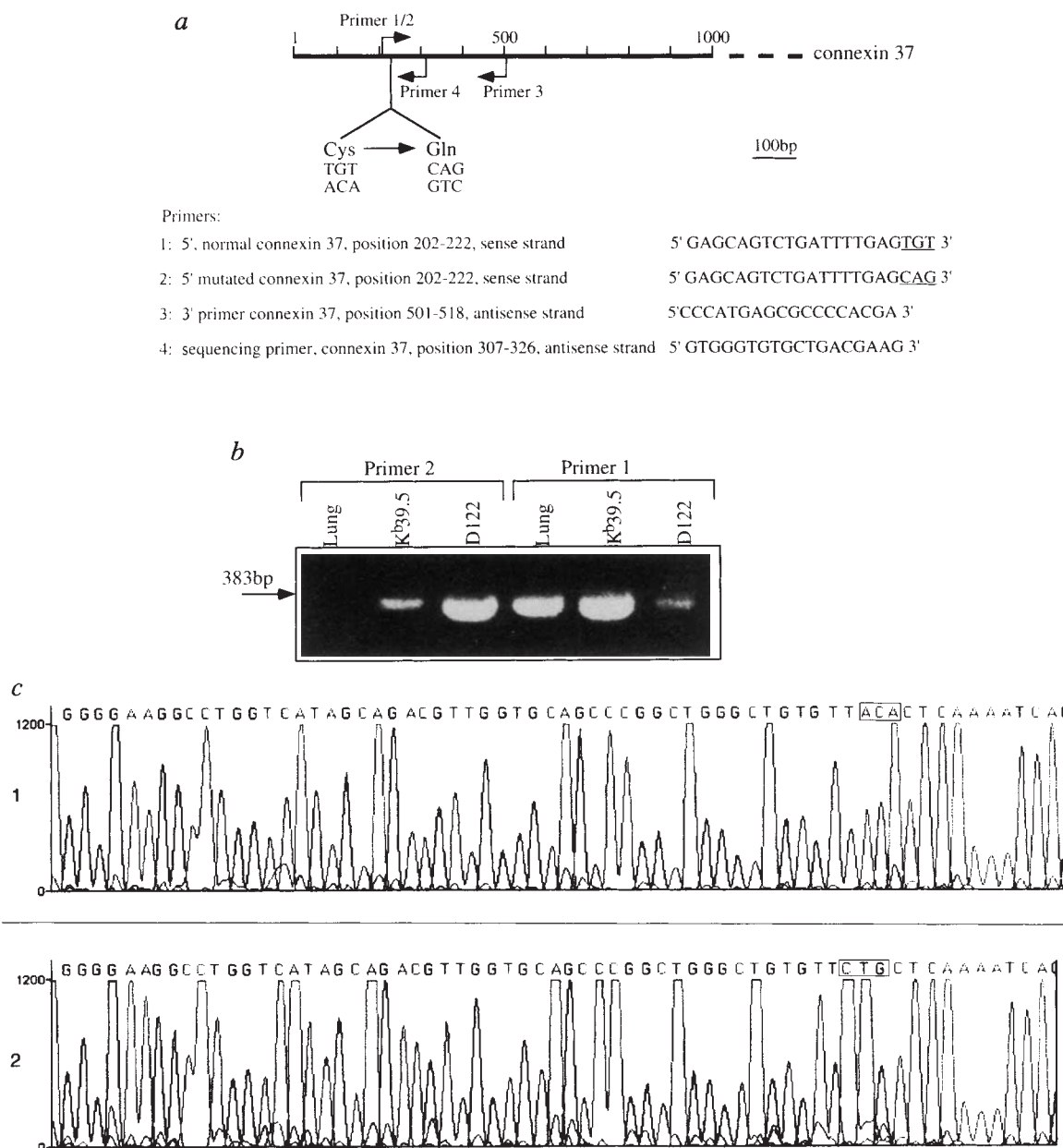


FIG. 4 PCR amplification and sequences of connexin 37 cDNA in 3LL clones and in normal lungs. **a**, Schematic presentation of connexin 37 showing the Cys-to-Gln mutation site and the primers used for amplification and sequencing. **b**, PCR products amplified from lung, K^b39.5 and D122 cDNAs using primers 1 + 3 (marked as primer 1), or primers 2 + 3 (marked as primer 2). **c**, Sequencing of PCR products amplified by primers 1 + 3 from lung, K^b39.5 and D122 (panel 1), and by primers 2 + 3 from K^b39.5 and D122 (panel 2). Only one example of each of the sequences is shown.

METHODS. Isolation of total RNA was as described⁸. Reverse transcrip-

tion was on 2 µg of total RNA with an oligo(dT) primer. cDNA corresponding to 100 ng total RNA was annealed to primers 1 + 3, or 2 + 3 at 56 °C and amplified for 30–35 cycles by PCR. The PCR products were separated on 1.5% agarose gels. Excised bands were extracted using a Gene-Clean II kit as recommended by the manufacturer (Bio 101 Inc, LaJolla). Purified PCR products, 150 ng per sample, were directly sequenced (with sequencing primer 4) using the Applied Biosystem Taq Dyeoxy Terminator Cycle Sequencing Kit 401113 on a 373A DNA Sequencer.

4. Rotzschke, O. et al. *Nature* **348**, 252–254 (1990).
5. Van Bleek, G. M. & Nathenson, S. G. *Nature* **348**, 213–216 (1990).
6. Udaka, K., Tsomides, T. J. & Eisen, N. H. *Cell* **69**, 989–998 (1992).
7. Rammensee, H. G., Falk, K. & Rotzschke, O. A. *Rev. Immun.* **11**, 213–244 (1993).
8. Plaksin, D., Gelber, C., Vadai, E., Feldman, M. & Eisenbach, L. *Proc. natn. Acad. Sci. U.S.A.* **85**, 4463–4470 (1988).
9. Mandelboim, O., Feldman, M. & Eisenbach, L. *J. Immun.* **148**, 3666–3673 (1992).
10. Plaksin, D., Gelber, C. & Eisenbach, L. *Int. J. Cancer* **52**, 771–777 (1992).
11. Franks, L. M. et al. *Br. J. Cancer* **49**, 423–429 (1984).
12. Fremont, D. H., Matsumura, M., Stura, E. A., Peterson, P. A. & Wilson, I. A. *Science* **257**, 919–927 (1992).
13. Zhang, W., Young, A. C. M., Imarai, M., Nathenson, S. G. & Sacchettini, J. C. *Proc. natn. Acad. Sci. U.S.A.* **89**, 8403–8407 (1992).
14. Levitt, M. J. *molec. Biol.* **170**, 723–764 (1983).
15. Huang, M. M., Amheim, N. & Goodman, M. F. *Nucleic Acids Res.* **20**, 4567–4573 (1991).
16. Willecke, K. et al. *J. Cell Biol.* **114**, 1049–1057 (1991).
17. Willecke, K., Hennemann, H., Dahl, E., Jungblute, S. & Heynkes, R. *Eur. J. Cell Biol.* **56**, 1–

- 7 (1991).
18. Lee, S. W., Tomasetto, C. & Sager, R. *Proc. natn. Acad. Sci. U.S.A.* **88**, 2852–2859 (1991).
19. Lee, S. W., Tomasetto, C., Paul, D., Keyomarsi, K. & Sager, R. *J. Cell Biol.* **118**, 1213–1221 (1992).
20. Zhu, D., Kidder, G. M., Caveney, S. & Naus, C. C. *Proc. natn. Acad. Sci. U.S.A.* **89**, 10218–10221 (1992).
21. Eghabali, B., Kessler, J. A., Reid, L. M., Rou, C. & Spray, D. C. *Proc. natn. Acad. Sci. U.S.A.* **88**, 10701–10705 (1991).
22. Townsend, A. et al. *Nature* **340**, 443–448 (1989).
23. Falk, K. et al. *J. exp. Med.* **174**, 425–434 (1991).

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Picornaviral 3C cysteine proteinases have a fold similar to chymotrypsin-like serine proteinases

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THE picornavirus family includes several pathogens such as poliovirus, rhinovirus (the major cause of the common cold), hepatitis A virus and the foot-and-mouth disease virus. Picornaviral proteins are expressed by direct translation of the genomic RNA into a single, large polyprotein precursor^{1,2}. Proteolysis of the viral polyprotein into the mature proteins is assured by the viral 3C enzymes, which are cysteine proteinases³⁻⁶. Here we report the X-ray crystal structure at 2.3 Å resolution of the 3C proteinase from hepatitis A virus (HAV-3C). The overall architecture of HAV-3C reveals a fold resembling that of the chymotrypsin family of serine proteinases, which is consistent with earlier predictions^{7,8}. Catalytic residues include Cys 172 as nucleophile and His 44 as general base. The 3C cleavage specificity for glutamine residues is defined primarily by His 191. The overall structure suggests that an intermolecular (*trans*) cleavage releases 3C and that there is an active proteinase in the polyprotein.

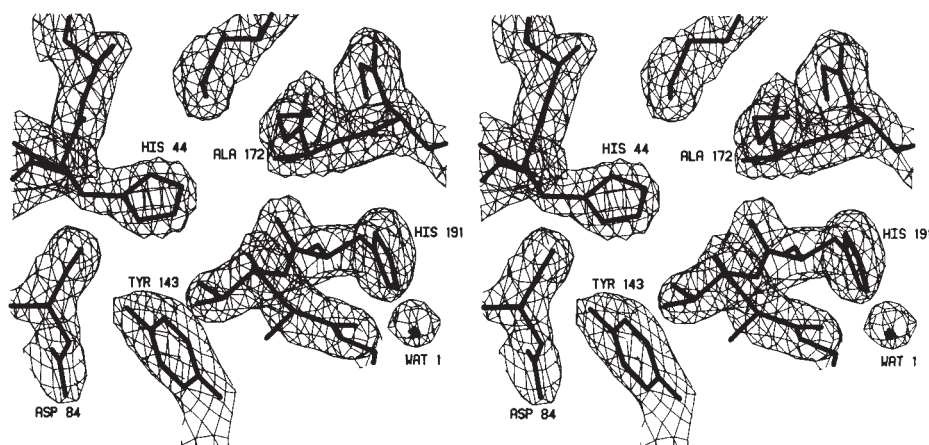
FIG. 1 Stereographic view of a portion of the electron density map obtained at 2.3 Å resolution for the HAV-3C proteinase structure. The region chosen surrounds the mutated Ala 172 which in the wild-type is the active-site cysteine. Bonds between protein atoms are represented by thick solid lines; the water molecule is represented by a small cross. The electron density corresponds to a SIGMAA-weighted¹⁷ $(2|F_o| - |F_c|)\exp(i\Phi_c)$ map contoured at 0.35 e Å⁻³ ($|F_o|$ and $|F_c|$ are the measured and calculated structure factor amplitudes respectively; Φ_c are the calculated phases from the refined model).

METHODS. Double mutant of HAV-3C proteinase (Cys 24 Ser, Cys 172 Ala) was expressed, purified and crystallized as described⁹. Crystals belong to space group P6₃22 ($a = b = 65.2$ Å, $c = 246.1$ Å), with one molecule per asymmetric unit and a solvent content of 61%. Synchrotron diffraction data were collected for the native crystal and 7 heavy-atom derivatives (10 mM NaAuCl₂, 100 mM trimethyl lead acetate, 10 mM K₂PtCl₄, 100 mM trimethyl lead acetate +10 mM K₂Pt(CN)₄, 10 mM (CH₃COO)₃Eu, 10 mM *p*-chloromercuribenzenesulphonate, 10 mM K₂Pt(CN)₄; soaking times, 20–40 h) to a maximum resolution of 2.3 Å on beamline station 6A2 (ref. 18) at the Photon Factory, Tsukuba, Japan. Imaging plates were processed using the WEIS program¹⁹. The native data set is 93% complete ($I > 0$; 80% for 2.4–2.3 Å) with a 6/1 ratio of observations to unique reflections merging to 6.8% ($\sum_o ||I_o - \bar{I}|/\sum_o I_o$, 5.2–7.2% for all data sets). Initial heavy-atom sites were obtained using Patterson vector superposition techniques included in SHELXS-90 (ref. 20) corroborated with Harker sections and cross-difference Fourier maps between heavy-atom derivatives. Heavy-atom sites, including anomalous occupancy, were refined with the program ML-PHARE²¹. The MIRAS phases had an overall figure of merit of 0.61 to 2.8 Å resolution

For crystallographic work we used a double mutant form of HAV-3C in which the two cysteine residues were replaced by site-directed mutagenesis (at residue 24 (Cys 24 Ser) and at residue 172 in the active site (Cys 172 Ala))⁹. The HAV-3C model has now been refined to a present *R*-factor of 20.9% (20–2.3 Å, $|F_o| > 1\sigma|F_o|$) with root-mean-square deviations on bond lengths and bond angles of 0.024 Å and 3.5°, respectively. A portion of the $(2|F_o| - |F_c|)$ electron density map in the region of the active site is shown in Fig. 1. The overall architecture of HAV-3C consists of two distinct domains, each having antiparallel β -barrel topology (Fig. 4a). These β -barrels are composed of two orthogonally packed β -sheets, as described for the serine proteinases¹⁰. The assignment of the secondary structural features of HAV-3C is shown in Figs 4b and 2a.

The colour-coded comparison of the three-dimensional structure of HAV-3C with three representatives of the chymotrypsin-like serine proteinases (Fig. 2b) indicates that all of these proteinases have a common fold. The overall fold predicted for the 3C proteinases^{7,8} is observed in HAV-3C. Topographically equivalent residues between HAV-3C and the chymotrypsin-like serine proteinases (Fig. 2a) are found mainly in the β -strands defining the cores of the two antiparallel β -barrels. The stretch of residues connecting strands B₂ and C₂ that helps define specificity adopts an extended hairpin-turn conformation in HAV-3C which is similar to the equivalent loop in the bacterial α -lytic proteinase¹¹. The C-terminal helix of the chymotrypsin-like serine proteinases (missing in the sindbis virus core protein¹²) is present in the HAV-3C proteinase. The N-terminal helix is a unique feature of HAV-3C not found in any of the other serine proteinases. Our three-dimensional structure of HAV-3C, in which the N and C termini are distant from the active site (Fig. 4a, b), makes it improbable that the cleavage releasing the proteinase from the polyprotein is intramolecular (*cis*).

Comparison of the active-site geometry of HAV-3C with that of chymotrypsin¹³ and papain¹⁴ (Fig. 3) indicates that the picor-



with phasing power of 0.5–1.4. The currently refined model includes residues 1–212 and 6 water molecules. Residues 50–53 and 146–153 are included as polyalanine and the last 5 C-terminal residues are disordered (the recombinant HAV-3C used here has a total of 217 residues, which is two residues shorter at the C-terminus than the proposed cleavage site²²). The model has been refined by a combination of simulated annealing using XPLOR²³, restrained parameter least-squares refinement with PROLSQ²⁴ and TNT²⁵ (including the bulk solvent correction), and visual inspection of the SIGMAA-weighted¹⁷ $(2|F_o| - |F_c|)$ and $(|F_o| - |F_c|)$ electron density maps using FRODO²⁶. The model is consistent in having solvent-accessible heavy-atom binding sites (like histidine and methionine residues) adjacent to the 8 different heavy-atom positions. The model is in agreement with the most favoured regions of the Ramachandran plot²⁷ and the 3D profile²⁸. Coordinates of the full refined model of HAV-3C proteinase will be deposited with the Protein Data Bank, Brookhaven.

naviral cysteine proteinases share catalytic features with the chymotrypsin-like proteinases¹⁵ but are distinct from the eukaryotic cysteine proteinases. The nature of the nucleophilic Cys 172 and the general base His 44, inferred from earlier predictions^{7,8} and supported by site-directed mutagenesis experiments^{4,6}, is now confirmed by the structure of HAV-3C. The conformation of the oxyanion pocket in HAV-3C (Fig. 3a, residues 169–172)

differs from that in chymotrypsin (Fig. 3b, residues 192–195). Residues 169–172 adopt a 3_10 helical conformation in HAV-3C by forming an additional hydrogen bond between the carbonyl oxygen of Pro 169 and the amide nitrogen of Ala 172. Replacement of Ala 172 by a cysteine having $\chi_1 = -60^\circ$, as found in the serine proteinases, results in a steric clash involving the sulphur and the carbonyl oxygen of Pro 169 (distance 2.9 Å). In wild-

FIG. 2 a, Sequence alignment of HAV-3C with the serine proteinases chymotrypsin (CHYT)¹³, α -lytic proteinase (α LP)¹¹ and sindbis core protein (SCP)¹² based on topographically equivalent residues in three-dimensional structures. An initial orientation matrix calculated with program O (T. Alwyn Jones, personal communication) used the C_α atoms of His 44, Asp 84, Ala 172 of HAV-3C and those of the catalytic triad residues of the serine proteinases. The 'LSQ IMPROVE' included in the program was then used to define the topographically equivalent residues if at least three contiguous residues between the two structures had C_α atom distances below a given cutoff. The upper- and lower-case letters in the serine proteinase sequences represent topographically equivalent residues using a distance cutoff of 2.5 Å and 3.8 Å, respectively. Bold letters highlight conserved residues, including the nucleophilic cysteine in HAV-3C or the serine residue in the serine proteinases. In numbering the serine proteinases (CHYT #, α LP #, SCP #), we recorded the first and last residues of each stretch of topographically equivalent residues. The number in parentheses between two segments of topographically equivalent residues represents the difference in the number of residues found in each of the serine proteinases minus the number of residues found in HAV-3C between the two segments (when the space is limited, this value is replaced by a vertical bar and the value is given in parentheses after the first residue number of the second segment). The HAV-3C # numbering includes the two mutations Cys 24 Ser and Cys 172 Ala. The line Sec.St-3D indicates the secondary structure. Symbols: $\leftarrow$, α -helix; $||$, β -strand; ~, disordered region according to the electron density map; x, residues absent in recombinant HAV-3C proteinase. Sec.St-G and Sec.St-BF indicate secondary structure predictions (refs 7 and 8 respectively). b, Colour-coded comparison of the overall structure of HAV-3C with the serine proteinases chymotrypsin¹³, α LP¹¹ and SCP¹² (generated with SETOR³⁰). A different colour is used for each β -strand forming the two antiparallel β -barrels. The NH₂ domain and the COOH domain are at the top and bottom of each structure respectively. From the N terminus to the C terminus, the sequence of colour used for each domain is orange, green, yellow, blue, magenta and cyan. β -strands E_{1a} and E_{1b} in the HAV-3C structure have been assigned the same magenta colour. The C-terminal helix observed in these structures (except in SCP) are coloured red. The yellow balls and sticks represent side-chain atoms of the nucleophile serine or Ala 172 in HAV-3C (Cys 172 in wild-type) involved in catalysis. The blue balls and sticks represent the side chain of the general-base histidine involved in catalysis.

a

```

<--  $\alpha_N$  --> |-- A1--|~ |--- B1---| | C1 | ~~~~ |-- D1--| | E1a | | E1b |
aaaaaaaaaaaa aaaaaa bbbbbbbb ccccc dddd eeeeeeeeeee
1 aaaaaaaaaaaaaa s bbbbbbbccccccc ddddddd e
STLEIAGLVKRNVLQFVGVEKNGCVRVMMNALGVKDDWLLVPSHAYKFEKDYEMMEFYFNRGGTYYSISAGNVVQSILDV
(N-term).WPWQVSLQDK...tgfhFCGGSLINENWVVTAAHCG.....SDVVVAgigklki...akvf...
(N-term).....iEYSIn.....LCSVg...KGFVTAGHCGt.....tarig.....
(N-term)...RLFDVK.....vIGHALAm...kVMKPLHVK.....tid.....kft...

~ | F1 | domain connection |-- A2--| |--- B2---| |  $\beta_A$  | ~~~~ |  $\beta_B$  | |--
ffffffffff aaaaaa bbbbbbbb cccc
eeeeeeeeeeee ffffffffaaaaaa bbbbbbbb cccc
81 90 100 110 120 130 140 150 160
GFQDVLMLKVPITPKFRDITQHFIKKGDVPRALNRLATLVTTVNGTPTMLISEGPKMEEKATYVHKNDGTTVDLTVDQA
92 94|102(+7) 110 (+2) 122 129(-2)130-2|134(+1) 142(+7)154 165 (-8) 179
skyDITLLKLSt.....clPSASDD..FAAttcvttgwg..RLQQASLplsn.....AM
102 109(0)112 119(+8)122 125 (-4) 131 142(-3)156 166(-2)167-9 (-6) 173
...DRAWVSLt...qtll...rgst....avGAAVCRSGRT...GYQCGTtAKN..vta.....aegavrgL
163 167 (0) 176-8(-2)179-81(-5)183 192 (-8) 194 198 (-24)
...DMEFa.....eaf..TYT.....ehpegFYNWh.....GAVQY.....

C2--| | D2 | |-- E2--| | F2 | < $\alpha_C$ > ~~~~~xx Sec.St-3D
ddddd eeeeeee ffff aaaaaaa Sec.St-G.
c dddddd eeeeeeeee fffffff aaaaaaa Sec.St-BF
161 170 A 180 190 200 210 219
WRGKGEGLP $\mathbf{G}$ MCGGALVSSNQSIQNALGIHVAGGNSILVAKLVTOEMFQNDKKIESQ
184|188(+3) 201 (-1) 208 217|224(+5) 232(+2)238 240
ICAgvSSCMGDSGGPLVC.....tlVGIVSWGStPGVYARVt...vqq.(C-term)
201 (-4) 207 217|224(+13) 232
TQGNacMGRGDSGGSWIT.....gQAQGVMSGGN.RSSLFERLQ.....(C-term)
202 205|210(+3) 221 (-4) 225 235|240(+4)246
.rFTII.ggrGDSGRPIMd.....rvvaIVLGGADTALSVVT.....(C-term)

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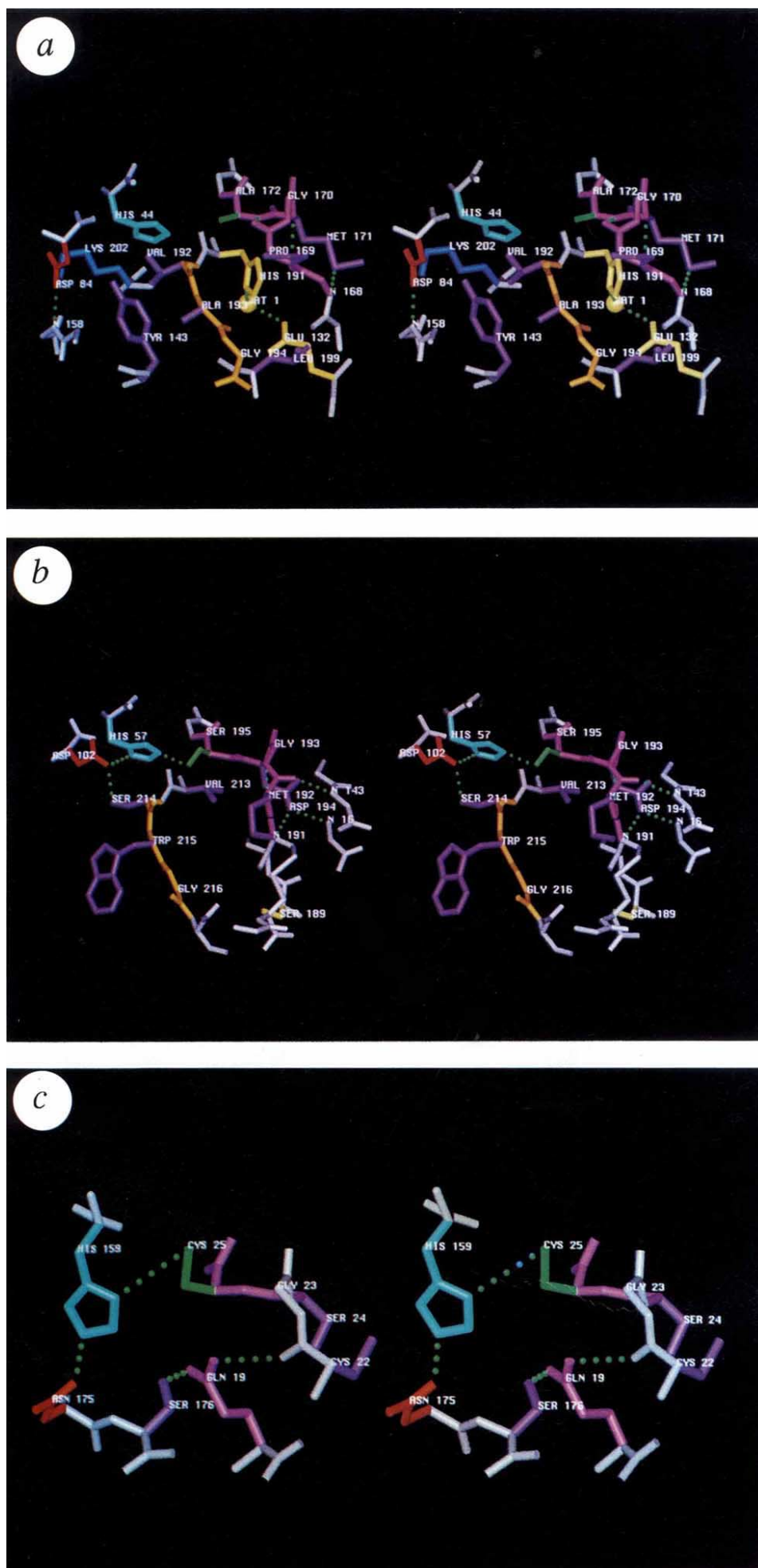
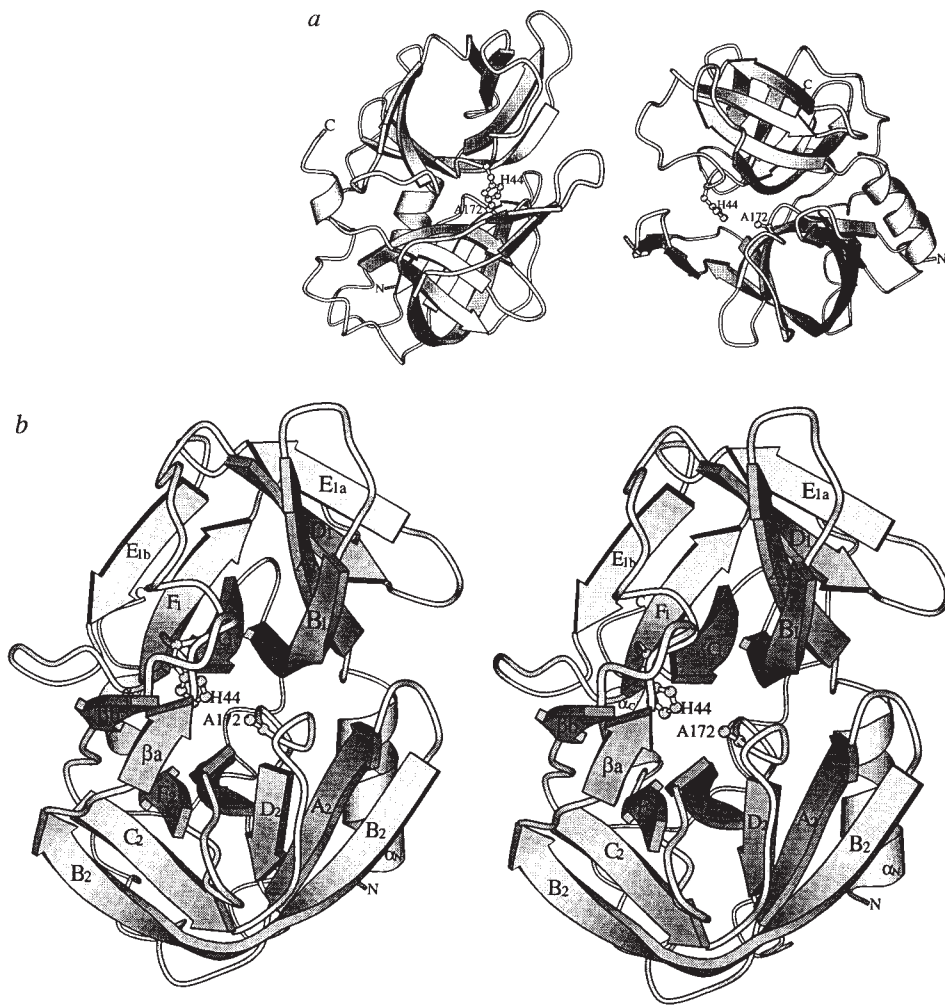


FIG. 3 Stereo representation of the active-site region of **a**, HAV-3C; **b**, chymotrypsin¹³; and **c**, papain¹⁴ (generated with SETOR³⁰). Colours highlight equivalent parts of the active site: green, side chain of the nucleophilic residue; cyan, side-chain of the general-base residue; red, side chain of the third member residue (not involved in HAV-3C, see text); pink, oxyanion hole; magenta, side chain of residues in the active site region. For HAV-3C and chymotrypsin: orange, β -strand peptide binding; yellow, P_1 -specificity pocket. Important hydrogen bonds are shown as green dotted lines.

FIG. 4 a, Overall architecture of HAV-3C proteinase structure in two different orientations (generated with MOLSCRIPT²⁹). The NH₂ domain and the COOH domain are shown at the top and bottom of each orientation respectively. Left, the antiparallel β -barrel of the NH₂ domain and the two orthogonally packed β -sheets of the COOH domain are evident; right, the antiparallel β -barrel of the COOH domain and the two orthogonally packed β -sheets of the NH₂ domain can be seen. Side chains of the active-site residues His 44 and Ala 172 (Cys 172 in wild type) are also shown. b, Stereographic representation of the HAV-3C proteinase structure (generated with MOLSCRIPT²⁹). Secondary structure is assigned according to the chymotrypsin-like serine proteinases. Subscripts 1 and 2 refer to the NH₂ and COOH domains, respectively.



type HAV-3C, the sulphur of the active-site residue may prevent residues 169–172 from adopting this 3_{10} helical conformation. It is likely that the overall conformation of the oxyanion hole in the active wild-type enzyme will be similar to that of the chymotrypsin-like serine proteinases.

The Asp 84 of HAV-3C (equivalent to Asp 102, the third member of the catalytic triad in chymotrypsin) does not interact with His 44 as predicted⁷ (Fig. 3*a, b*). Instead, the carboxylate side chain is in strong electrostatic interaction with the side chain of Lys 202 (distance 3.0 Å), and it makes a hydrogen bond with the main-chain amide of Asp 158 (2.6 Å). Because of the nature of these interactions, we think that the conformation of Asp 84 will remain in the wild-type enzyme of HAV-3C. In the present structure, the N_{δ1} of His 44, although it is 3.2 Å from the hydroxyl oxygen of Tyr 143, is not oriented properly to form a hydrogen bond. The role of the third member in the catalytic triad, which involves a cysteine as the nucleophilic residue of the papain-like enzyme, is not fully understood. In papain, the third member is an asparagine residue, which would be likely to influence the orientation of the side chain of the histidine (Fig. 3*c*). The charge effect is not essential, probably because abstraction of a proton from a sulphhydryl group to create a nucleophilic thiolate anion is more favourable than removing it from a hydroxyl group (pK_a 8.3 for R-SH, compared with >15.0 for R-OH). We suggest that the catalytic apparatus of HAV-3C proteinase involves only a dyad of cysteine/histidine residues. It may also be that Tyr 143 in HAV-3C contributes some steric restraint to help position the His 44 side chain.

The specificity of cleavage by the 3C proteinases is for peptide bonds between glutamine and residues with small side chains^{1,2,16}. HAV-3C must recognize the carbonyl oxygen atom

of the glutamine amide because *N*, *N*-dimethylglutamine binds equally well to the enzyme (B.A.M. *et al.*, unpublished results). Earlier predictions^{7,8} correctly placed His 191 in the *S*₁ binding pocket of HAV-3C. Our structure reveals additional features associated with conferring glutamine specificity on the *P*₁ cleavage site which may be particular to HAV-3C. There is a buried water molecule (WAT 1 in Fig. 3a) within hydrogen-bonding distance of the N_{δ1} of His 191 and of the carboxyl group of the buried Glu 132 residue. Residues surrounding this region are predominantly hydrophobic (Leu 119, Ile 130, Leu 176, Leu 199, Ala 201), so it is likely that the buried carboxyl group of Glu 132 is protonated and neutral. The water could therefore act as hydrogen-bond acceptor from Glu 132 and donor to N_{δ1} of His 191; the imidazole ring of His 191 would then be neutral but still have the necessary hydrogen-bonding potential on N_{ε2} to donate a proton to the carbonyl oxygen of the amide side chain of a *P*₁ glutamine-containing substrate. The uncharged His 191 could thus help to discriminate between *P*₁ glutamine and *P*₁ glutamate, as has been observed in cleavage patterns of synthetic peptide substrates¹⁶. As Glu 132 is not conserved among the other picornaviral 3C proteinases, the glutamine preference for those enzymes must be achieved in some other but closely related manner. □

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1. Palmenberg, A. C. *Rev. Microbiol.* **44**, 603–623 (1990).
2. Dougherty, W. G. & Semler, B. L. *Microbiol. Rev.* **57**, 781–822 (1993).
3. Argos, P., Kamer, G., Nicklin, M. J. H. & Wimmer, E. *Nucleic Acids Res.* **12**, 7251–7267 (1984).
4. Ivanoff, L. A., Towatari, T., Ray, J., Korant, B. D. & Petteway, S. R. *J. Proc. natn. Acad. Sci. U.S.A.* **83**, 5392–5396 (1986).
5. Hämmerle, T., Hellen, C. U. T. & Wimmer, E. *J. biol. Chem.* **266**, 5412–5416 (1991).

6. Kean, K. M., Teterina, N. L., Marc, D. & Girard, M. *Virology* **181**, 609–619 (1991).
7. Gorbalenya, A. E., Donchenko, A. P., Blinov, V. M. & Koonin, E. V. *FEBS Lett.* **243**, 103–114 (1989).
8. Bazan, J. F. & Fletterick, R. J. *Proc. natn. Acad. Sci. U.S.A.* **85**, 7872–7876 (1988).
9. Cherniaia, M. M., Malcolm, B. A., Allaire, M. & James, M. N. G. *J. molec. Biol.* **234**, 890–893 (1993).
10. Chothia, C. & Janin, J. *Biochemistry* **21**, 3955–3965 (1982).
11. Fujinaga, M., Delbaere, L. T. J., Brayer, G. D. & James, M. N. G. *J. molec. Biol.* **183**, 479–502 (1985).
12. Tong, L., Wengler, G. & Rossmann, M. G. *J. molec. Biol.* **230**, 228–247 (1993).
13. Fujinaga, M. et al. *J. molec. Biol.* **195**, 397–418 (1987).
14. Drenth, J., Kalk, K. H. & Swen, H. M. *Biochemistry* **15**, 3731–3738 (1976).
15. Kraut, J. et al. *Cold Spring Harbor Symp. quant. Biol.* **36**, 117–123 (1971).
16. Jewell, D. A., Swietnicki, W., Dunn, B. M. & Malcolm, B. A. *Biochemistry* **31**, 7862–7869 (1992).
17. Read, R. J. *Acta. crystallogr.* **A42**, 140–149 (1986).
18. Sakabe, N. *Nucl. Inst. Meth. Phys. Res.* **A303**, 448–463 (1991).
19. Higashi, T. J. *appl. Crystallogr.* **22**, 9–18 (1989).
20. Sheldrick, G. M. *Acta. crystallogr.* **A46**, 467–473 (1990).
21. Otwinowski, Z. *CCP4 Study Weekend* 80–86 (Daresbury, UK, 1991).
22. Cohen, J. I., Ticehurst, J. R., Purcell, R. H., Buckler-White, A. & Baroudy, B. M. *J. Virol.* **61**, 50–59 (1987).
23. Brünger, A. T. *X-PLOR Version 3.1 Manual* (Yale Univ. Press, New Haven and London, 1992).
24. Hendrickson, W. A. *Meth. Enzym.* **115**, 252–270 (1985).
25. Tronrud, D. E., TenEyck, L. F. & Matthews, B. W. *Acta crystallogr.* **A43**, 489–503 (1987).
26. Jones, T. A. *Meth. Enzym.* **115**, 157–171 (1985).
27. Morris, A. L., MacArthur, M. W., Hutchinson, E. G. & Thornton, J. M. *Proteins* **12**, 345–364 (1992).
28. Lüthy, R., Bowie, J. U. & Eisenberg, D. *Nature* **356**, 83–85 (1992).
29. Kraulis, P. J. *J. appl. Crystallogr.* **24**, 946–950 (1991).
30. Evans, S. V. *J. molec. Graphics* **11**, 134–138 (1993).

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Membrane targeting of the small GTPase Rab9 is accompanied by nucleotide exchange

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THE Rab GTPases are key regulators of vesicular transport^{1–6}. A fraction of Rab proteins is present in the cytosol, bound with GDP, complexed to a protein termed GDI^{7–10}. Rab9 is localized primarily to late endosomes, where it aids the transport of mannose 6-phosphate receptors to the *trans*-Golgi network¹¹. It has been proposed that Rab proteins are delivered to specific membranes by GDI, and that this process is accompanied by the exchange of bound GDP for GTP^{1–3}. In addition, Rab localization requires carboxy-terminal prenylation and specific structural determinants^{12–14}. Here we describe the reconstitution of the selective targeting of prenylated Rab9 protein onto late endosome membranes and show that this process is accompanied by endosome-triggered nucleotide exchange.

To reconstitute Rab protein recruitment, we used prenylated Rab9–GDI complexes isolated in crude form from the cytosol of Chinese hamster ovary (CHO) cells expressing Rab9 at 50-fold higher levels than untransfected cells. Alternatively, they were reconstituted using purified, prenylated Rab9 and GDI. Three potential membrane targets were tested: mannose 6-phosphate receptor (MPR)-enriched late endosomes, endoplasmic reticulum (ER) membranes, and lysed red blood cell ghosts.

Prenylated Rab9, presented to MPR-enriched membranes as a partially purified, GDI-complex, became membrane-associated on incubation at 37 °C, but not 0 °C. (Fig. 1a). ER-derived mem-

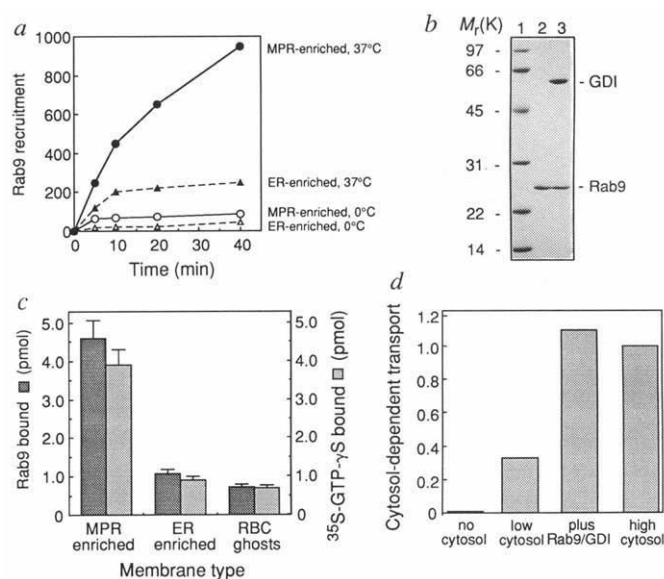


FIG. 1 Membrane recruitment of functional Rab9 is target-specific and blocked at 0 °C. **a**, Rab9 binding to MPR-enriched membranes (circles) or ER-enriched membranes (triangles) with GTP-γS at 37 °C (solid symbols) or 0 °C (open symbols). At 40 min, ~30% (1.5 ng) of the added Rab9 was recruited onto endosomal membranes. **b**, Coomassie blue stained, SDS-PAGE of purified prenylated Rab9 and Rab9–GDI complexes. Lane 1, size markers; lane 2, prenylated Rab9 (1 μg); lane 3, prenylated Rab9–GDI complex (3 μg). **c**, Specificity of nucleotide exchange and membrane recruitment of Rab9 protein. **d**, Rab9–GDI complexes (**b**) are active in endosome to *trans*-Golgi network transport *in vitro*.

METHODS. **a**, Reactions (250 μl) contained 30 mM HEPES, pH 7.2, 40 mM sucrose, 90 mM KCl, 10 mM NaCl, 3.5 mM MgCl₂, 0.3 mM EDTA, 0.4 mM GTP-γS, and 50 mM (NH₄)₂SO₄, 1× ATP-regeneration system¹⁵, 1× protease inhibitor cocktail¹⁵, 3 μg membranes, and ~5 ng Rab9 as a crude Rab9–GDI complex (30 μg total protein). Reactions were stopped by addition of ice-cold buffer (64 mM HEPES, pH 8.0, 100 mM NaCl, 8 mM MgCl₂, 2 mM EDTA), followed by centrifugation at 95,000 r.p.m. for 10 min at 2 °C. Membrane pellets were washed, and analysed by SDS-PAGE and anti-Rab9 immunoblotting⁸. Crude Rab9–GDI complexes of *M*_r 80K were obtained by gel filtration⁸. Membranes were obtained from a rat liver postnuclear supernatant as described²⁵, with minor modifications. Endosome- or MPR-enriched membranes (detected by anti-MPR and anti-Rab9 immunoblotting) were collected at the 0.5–0.9 M sucrose interface; ER-enriched membranes (detected by anti-protein disulphide isomerase immunoblotting) were collected from the 1.3–1.5 M sucrose interface. **b**, Purified Rab9–GDI complexes were reconstituted with 95% efficiency by dialysing pure, prenylated Rab9 with an equimolar amount of pure GDI¹⁸, followed by Sephacryl S100 gel-filtration chromatography to remove CHAPS. Prenylated Rab9 was purified to >90% homogeneity from membranes of baculovirus-infected cells by solubilization in 2% CHAPS, Sephacryl-S100 gel filtration, and mono-Q anion-exchange chromatography in 1% CHAPS. **c**, Reactions were as in **a** in 100 μl, with 10 μg membranes and 500 ng Rab9 as a purified complex with GDI. Reactions contained 2 μM GTP-γS and 90.02 μM ³⁵S-GTP-γS (5 μCi), 100 mM (NH₄)₂SO₄ and no ATP-regeneration system. ³⁵S-GTP-γS radioactivity bound to proteins was assayed for half the sample by filter binding²⁰. The amount of Rab9 associated with membranes was monitored for the other half as in **a**. Red blood cell (RBC) ghosts were obtained as described²⁶. Low-molecular-size markers were from BioRad. **d**, *In vitro* transport¹⁵ was for 120 min with no cytosol, low cytosol (0.35 mg ml⁻¹) or low cytosol + 5 ng Rab9 complexed with equimolar GDI. Transport measured in the absence of cytosol was subtracted to yield cytosol-dependent transport¹¹. Cytosol yielded a 2.3-fold stimulation in total transport, and the standard error was 14% for the values shown.

branes bound less at both temperatures. Binding was both specific and efficient: as much as 30–50% of the added Rab9 protein became membrane-associated after 40 min. Purified, prenylated Rab9–GDI complexes (Fig. 1b) were recruited with similar high efficiency and specificity (Fig. 1c and Table 1). Red blood cell

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recruitment equalled late endosome recruitment at 0 °C and may reflect unstable Rab9-GDI complexes (Table 1); the difference in recruitment between ER and red blood cells represents contaminating endosomes in the ER fraction, because it contained low levels of MPRs and Rab9 (10–15% of the level in the late endosomes).

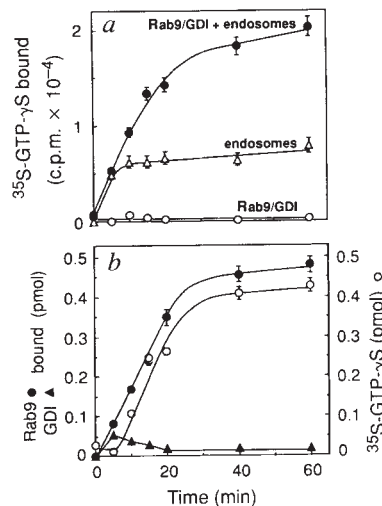


FIG. 2 a, Endosomal membranes stimulate GTP binding to membrane-associated Rab9 protein. Recruitment reactions were done using purified Rab9-GDI complexes and endosomal membranes, either alone or in combination. Reactions were analysed for the binding of ^{35}S -GTP- γS to the indicated components. b, Kinetics of Rab9 (●) and GDI (▲) membrane association, and membrane-triggered, Rab9 ^{35}S -GTP- γS binding (○). Error bars lie beneath the filled triangles in b. METHODS. Reactions were as described in Fig. 1c legend except that they contained 3 μg MPR-enriched membranes and 100 ng Rab9 in the form of a highly purified complex with GDI. Membrane-associated GDI was monitored by quantitative anti-GDI immunoblotting. The amount of Rab9-independent, membrane-associated ^{35}S -GTP- γS was subtracted to obtain the curve shown (○).

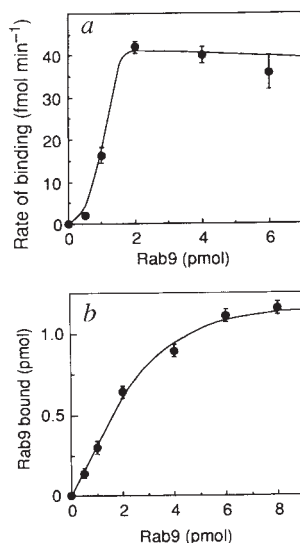


FIG. 3 The initial rate and extent of Rab9 recruitment onto MPR-enriched membranes are saturable. a, Initial rates of Rab9 targeting to MPR-enriched membranes were obtained from standard reactions done for 2 min with the indicated amounts of Rab9 in the form of highly purified Rab9-GDI complexes. b, Total Rab9 recruitment was monitored in standard reactions done for 40 min, with up to 8 pmol purified Rab9-GDI complex. Maximum Rab9 binding corresponded to about 1.2 pmol, ~50-fold over the amount of endogenous Rab9 present on the MPR-enriched membranes.

About 1 mol ^{35}S -GTP- γS became membrane-associated for each mol of membrane-bound, prenylated Rab9 protein for all membranes tested (Fig. 1c). Thus, Rab9 membrane association was accompanied by nucleotide exchange. This is a likely prerequisite for vesicular transport because a large fraction of Rab9 became membrane-associated, and purified Rab9-GDI complexes were functional; the complexes yielded maximal stimulation of the *in vitro* transport of MPRs from late endosomes to the *trans*-Golgi network¹⁵ assayed under comparable conditions (Fig. 1d).

Rab9 GTPase activity is likely to be stimulated by a cognate GAP protein^{16–18}. Rab9-GDP would then be a target for GDI-mediated membrane dissociation. GTP- γS stabilized (or enhanced) the membrane association of Rab9 with MPR-enriched membranes relative to reactions containing GTP, but had much less of an effect with an equivalent amount of an inappropriate target. The specificity of Rab9 recruitment was confirmed by the inability of prenylated Rab3A-GDI complexes to be recruited onto late endosomes (Table 1). Membrane surface proteins and Rab9 prenylation were required, but ATP hydrolysis was not. In addition, cytosol was unessential but was slightly stimulatory; high concentrations partially inhibited recruitment, perhaps due to free GDI (see below). Recruitment was inhibited by GDP, which may drive the equilibrium of the reaction strongly towards the Rab9-GDP-GDI complex. Finally, unoccupied bovine brain GDI strongly inhibited Rab9 recruitment, even in the presence of GTP- γS . This suggests that GDI may inhibit by direct interaction with membrane components involved in Rab recruitment.

Rab9 recruitment was associated with equimolar GTP binding (Fig. 1c). Immunoprecipitation confirmed that the GTP was bound directly to Rab9 protein (not shown). To determine whether this nucleotide-exchange process was stimulated by MPR-enriched membranes, we measured the rates of nucleotide

TABLE 1 Rab9 recruitment requires protease-sensitive membrane components and is inhibited by unoccupied GDI

Reaction condition	Rab9 recruitment	
	Crude system	Purified system
Complete	1.00	1.00
ER membranes	0.25	0.23
Erythrocyte ghosts		0.16
MPR membranes		
80 °C	0.17	
proteinase K	0.03	
0 °C	0.17	
Non-prenylated Rab9	0.00	
Prenylated Rab3A on:		
MPR membranes	0.02	
ER membranes	0.02	
Hexokinase	0.91	
Cytosol (0.5 mg ml $^{-1}$)	1.35	1.50
(1.0 mg ml $^{-1}$)	0.73	
GTP (0.4 mM)	0.70	
GDP (0.4 mM)	0.30	
GDI (5:1)	0.21	
(50:1)	0.14	
(500:1)	0.05	

Reactions contained GTP- γS as in Fig. 1 or as follows. Hexokinase: the ATP-regenerating system was replaced by an ATP-depleting system; GTP was substituted for GTP- γS ; GDP was substituted for GTP- γS in the absence of an ATP-regenerating system (which would regenerate GTP); cytosol was from CHO cells; unoccupied GDI was added to excess Rab9 at the indicated molar ratios. Non-prenylated Rab9 (5 ng) and prenylated Rab3A were also tested. Crude and purified systems refer to the purity of the Rab9-GDI complexes used. ATP-depleting system was 56 U ml $^{-1}$ hexokinase, 10 mM glucose. Unoccupied GDI was purified from bovine brain²⁴ and 50, 500 or 5,000 ng were added as indicated. MPR-enriched membranes were digested with proteinase K (100 μg ml $^{-1}$) for 10 min at 37 °C, before addition of 2 $\times$ protease inhibitor cocktail and 1 mM PMSF for 5 min on ice. Mock-digested membranes were treated first on ice with the protease inhibitors, followed by incubation at 37 °C with proteinase K. Non-prenylated Rab9 was purified from an *Escherichia coli* overproducing strain²⁰. Prenylated Rab3A was obtained by *in vitro* transcription/translation of a complementary DNA clone encoding Rab3A (provided by R. Scheller) in the presence of geranylgeranyl diphosphate⁸. Its recruitment efficiency and specificity is presented relative to *in vitro* translated, prenylated Rab9⁸.

exchange for purified Rab9-GDI complexes, late endosome membranes, or mixtures of these components, using a filtration assay (Fig. 2a). As expected, GDI blocked GTP- γ S binding to Rab9_{GDP}-GDI complexes over a 60-min incubation. MPR-enriched membranes showed some binding of GTP- γ S, probably due to other GTP-binding proteins. However, addition of purified Rab9-GDI complexes to MPR-enriched membranes led to a significant increase in membrane-associated ³⁵S-GTP- γ S. The overall kinetics of Rab9 membrane association and Rab9-dependent ³⁵S-GTP- γ S binding were very similar (Fig. 2b). Nevertheless, at the earliest time points ³⁵S-GTP- γ S binding lagged behind the association of Rab9_{GDP} with membranes. In addition, some GDI was initially detected on the membranes. These data suggest that Rab9_{GDP}-GDI complexes associate transiently with membranes; after a short lag, GDI is released, GTP binds and Rab9 becomes stably associated with the membrane.

Rab proteins may possess distinct, saturable, target membrane-specific receptors¹. Alternatively, a protein catalysing nucleotide exchange could be localized to specific membrane compartments; after the release of GDI, a doubly prenylated Rab protein could jump into the adjacent membrane in its GTP-bound conformation. To distinguish between these possibilities, we examined whether the initial rate and/or extent of Rab recruitment were saturable.

Both the initial rate (Fig. 3a) and the overall extent (Fig. 3b) of Rab recruitment were saturable, but only at a ~50-fold excess of Rab protein relative to endogenous levels. Recruited Rab proteins were stably associated with the targets because, unlike a significant fraction of membrane-recruited ADP ribosylation factor (ARF)¹⁹, they could not be dislodged by up to 4 mM phosphatidylcholine liposomes at 0 °C (~750-fold excess over endosomal membrane lipid). The high capacity of membranes for Rab9 protein was consistent with the finding that 50-fold overexpression of Rab proteins does not lead to their mislocalization^{4,11}. These data strongly suggest that Rab proteins associate with a relatively abundant downstream target protein; nucleotide exchange/GDI displacement appear to be rate-limiting for targeting, and the saturable initial rate may reflect this membrane-catalysed process.

We have shown that prenylated Rab9_{GDP} can be transferred to its specific membrane target when presented by GDI. Rab9 recruitment is accompanied by nucleotide exchange. Comparison of the rates of GDP release and GTP binding observed here with those measured for free Rab9 protein²⁰ suggest that Rab recruitment may involve a catalytic GDI-displacement factor (GDF), rather than just a conventional nucleotide exchange factor (GDS)^{16,21,22} that would enhance the intrinsic rate of GDP

release from Rab9 protein. Rab proteins, with GTP bound, would then associate stably with another downstream protein, perhaps a component of the SNARE complex²³, to catalyse vesicle targeting and/or fusion. □

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1. Pfeffer, S. R. *Trends Cell Biol.* **2**, 41–46 (1992).
2. Zerial, M. & Stenmark, H. *Curr. Opin. Cell Biol.* **5**, 613–620 (1993).
3. Takai, Y., Kaibuchi, K., Kikuchi, A. & Kawata, M. *Int. Rev. Cytol.* **133**, 187–233 (1992).
4. Bucci, C. et al. *Cell* **70**, 715–728 (1992).
5. Van der Sluis, P. et al. *Cell* **70**, 729–740 (1992).
6. Plutner, H. et al. *J. Cell Biol.* **115**, 31–43 (1991).
7. Araki, S., Kikuchi, A., Hata, Y., Isomura, M. & Takai, Y. *J. Biol. Chem.* **265**, 13007–13015 (1990).
8. Soldati, T., Riederer, M. A. & Pfeffer, S. R. *Molec. Biol. Cell* **4**, 425–434 (1993).
9. Regazzi, R., Kikuchi, A., Takai, Y. & Wollheim, C. B. *J. Biol. Chem.* **267**, 17512–17519 (1992).
10. Ullrich, O. et al. *J. Biol. Chem.* **268**, 18143–18150 (1993).
11. Lombardi, D. et al. *EMBO J.* **12**, 677–682 (1993).
12. Chavrier, P. et al. *Nature* **353**, 769–772 (1991).
13. Dunn, B., Stearns, T. & Botstein, D. *Nature* **362**, 533–535 (1993).
14. Brennwald, P. & Novick, P. *Nature* **362**, 560–563 (1993).
15. Goda, Y. & Pfeffer, S. R. *Cell* **55**, 309–320 (1988).
16. Burstein, E. S. & Macara, I. G. *Proc. natn. Acad. Sci. U.S.A.* **89**, 1154–1158 (1992).
17. Walworth, N. C., Brennwald, P., Kabaceni, A. K., Garrett, M. & Novick, P. *Molec. cell. Biol.* **12**, 2017–2028 (1992).
18. Strom, M., Vollmer, P., Tan, T. J. & Gallwitz, D. *Nature* **361**, 736–739 (1993).
19. Helms, J. B., Palmer, D. J. & Rothman, J. E. *J. Cell Biol.* **121**, 751–760 (1993).
20. Shapiro, A. D., Riederer, M. A. & Pfeffer, S. R. *J. Biol. Chem.* **268**, 6925–6931 (1993).
21. Moya, M., Roberts, D. & Novick, P. *Nature* **361**, 460–463 (1993).
22. Burton, J., Roberts, D., Montaldi, M., Novick, P. & De Camilli, P. *Nature* **361**, 464–467 (1993).
23. Söllner, T. et al. *Nature* **362**, 318–324 (1993).
24. Sasaki, T. et al. *J. Biol. Chem.* **265**, 2333–2337 (1990).
25. Tabas, I. & Kornfeld, S. *J. Biol. Chem.* **254**, 11655–11663 (1979).
26. Fairbanks, G., Steck, T. L. & Wallach, D. F. H. *Biochemistry* **10**, 2606–2617 (1971).

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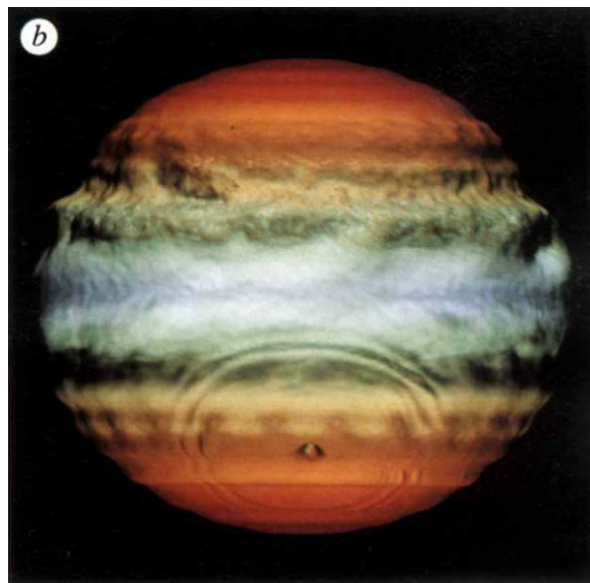
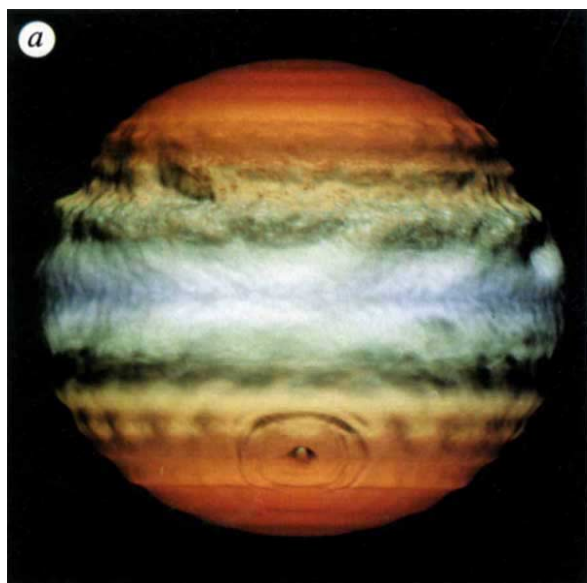
ERRATUM

Dynamic response of Jupiter's atmosphere to the impact of comet Shoemaker–Levy 9

Joseph Harrington, Raymond P. LeBeau Jr, Karl A. Backes & Timothy E. Dowling

Nature **368**, 525–527 (1994)

IN this letter, Fig. 2a and b was printed upside-down; the figure is correctly displayed here.



Imaging sequence space

Douglas C. Youvan

Digital imaging spectrophotometers can simultaneously measure the spectra of hundreds of features in a two-dimensional scene. While a variety of applications can be anticipated, a colorimetric analysis of mutants expressing pigmented proteins has already led to the development of efficient algorithms for optimizing combinatorial mutagenesis.

DEPENDING on the context, the acronym DIS can mean either a digital imaging spectrophotometer or digital imaging spectroscopy. DIS combines the techniques of optical spectroscopy and digital image processing so that a spectrum can be obtained for each pixel or group of pixels in a two-dimensional scene¹⁻³. Commercially available imaging spectrophotometers (Fig. 1) have now been developed that utilize charge coupled device (CCD) detectors and diffuse light sources in both a transmission and reflectance mode⁴. This instrument design, along with selective pixel processing within a defined feature, yields high resolution, low noise spectra from 400 nm to 1,000 nm. The quality of DIS spectra is far superior to that obtained from conventional spectrophotometers which cannot differentiate between absorption and light scattering. Furthermore, DIS is amenable to many different types of samples that cannot be easily analysed using conventional means.

Colourful targets

DIS has been extensively used in the study of microbial colonies directly from the surface of Petri dishes¹⁻⁷. In these analyses, DIS saved weeks of sample preparation time by dispensing with the need for such arduous tasks as picking colonies, resuspension of colonies in media, cellular disruption, centrifugation, and finally conventional spectroscopy of one sample at a time. DIS has already been used in botanical applications to investigate leaf and flower spectra, which could facilitate both ecology and plant molecular genetics studies. In natural products chemistry, DIS can be used to search for organisms that overproduce valuable pigments (for example, astoxanthin from yeast). In clinical microbiology, one might envisage the development of complex dye indicator media. Adaptation of DIS to a microscope has numerous uses in microbial ecology, including soil and water assays of microbes that cannot be cultured. DIS microscopy has potential applications in the field of histology, wherein one could use subtle spectral shifts to differentiate cellular and subcellular features in stained thin sections.

Combinatorial mutagenesis

DIS is extremely useful in protein mutagenesis studies if colorimetric indicators are available. Our work has focused on the ex-

pression of a pigment-protein complex in *Rhodobacter capsulatus*. The absorption spectrum of the bacteriochlorophyll in this photosynthetic organism has been used as a colorimetric reporter for the assembly of a light harvesting (LH) protein⁵⁻⁷. Rapid spectral measurements on thousands of colonies expressing mutagenized LH genes has created a unique situation in which DIS can be used to assay the outcome of another massively parallel technique, that of combinatorial mutagenesis. The ability of DIS software to sort and concisely encode spectra into a single 'pseudocoloured' contour plot (Fig. 2) enables one to assess the

efficiency of a given mutagenesis scheme.

Rather than confronting all of the structure and function questions associated with the design and synthesis of a single protein, combinatorial mutagenesis experiments rely on the ability to isolate a protein with desired properties (for example, binding specificity) out of a complex mixture or library of proteins. According to the exponential hypothesis⁷, it becomes increasingly improbable to find functional proteins in 'sequence space' as one mutagenizes more sites. Simultaneously mutagenizing 16 amino acid positions results in over 20¹⁶ possible sequences, a complexity much too great to be experimen-

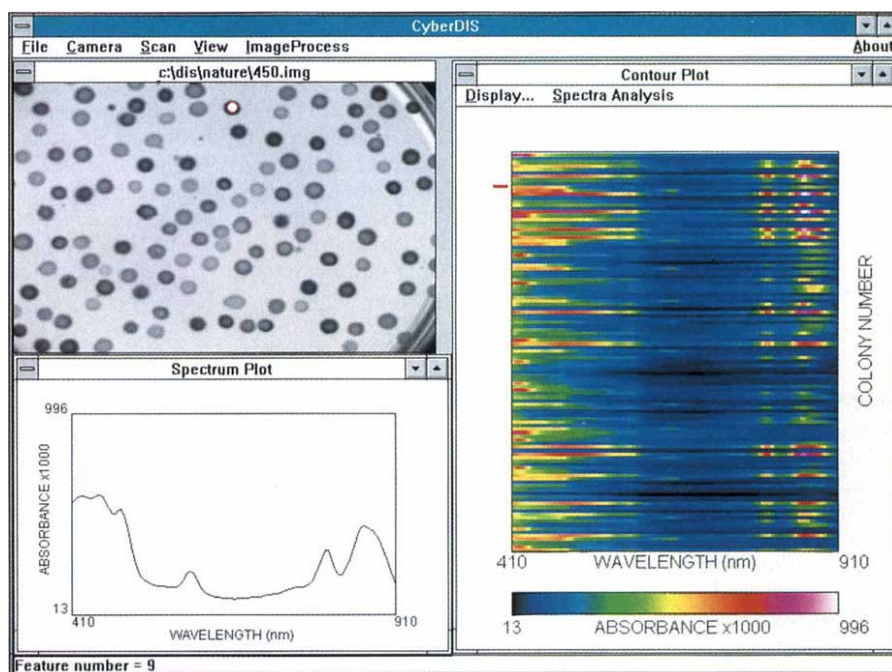


FIG. 1 The user interface of the ColonyImager digital imaging spectrophotometer (Kairos, Inc., Mountain View, California). The image of the Petri dish from which spectra were acquired is shown in the upper left window. The current feature of interest is marked by a filled white circle. The spectrum of this colony is plotted in the lower left window and its 'pseudocoloured' absorption spectrum is marked by the red bar next to the contour plot shown on the right. Each row of the contour plot represents a different colony whose x, y information is stored by the software. Similar to the spectrum in the conventional plot window, wavelength is represented on the horizontal axis. However, absorbance is now colour-coded according to the colour bar at the bottom. The contour plot has been scaled from black to white to represent the lowest and highest absorbances found in the entire dataset, respectively. The computer's mouse can be used to point to any colony in the image window or to any row in the contour plot with a real-time update of all three windows. One hundred and twelve spectra corresponding to colonies from nine different strains of *Rb. capsulatus* are represented in this figure. The spectrum plot of the colony selected shows absorption in the blue which is characteristic of a green mutant containing the carotenoid neurosporene. Spectral bands in the near-infrared are red-shifted forms of bacteriochlorophyll, which serve as spectroscopic reporters for the assembly of the light-harvesting pigment-protein complex.

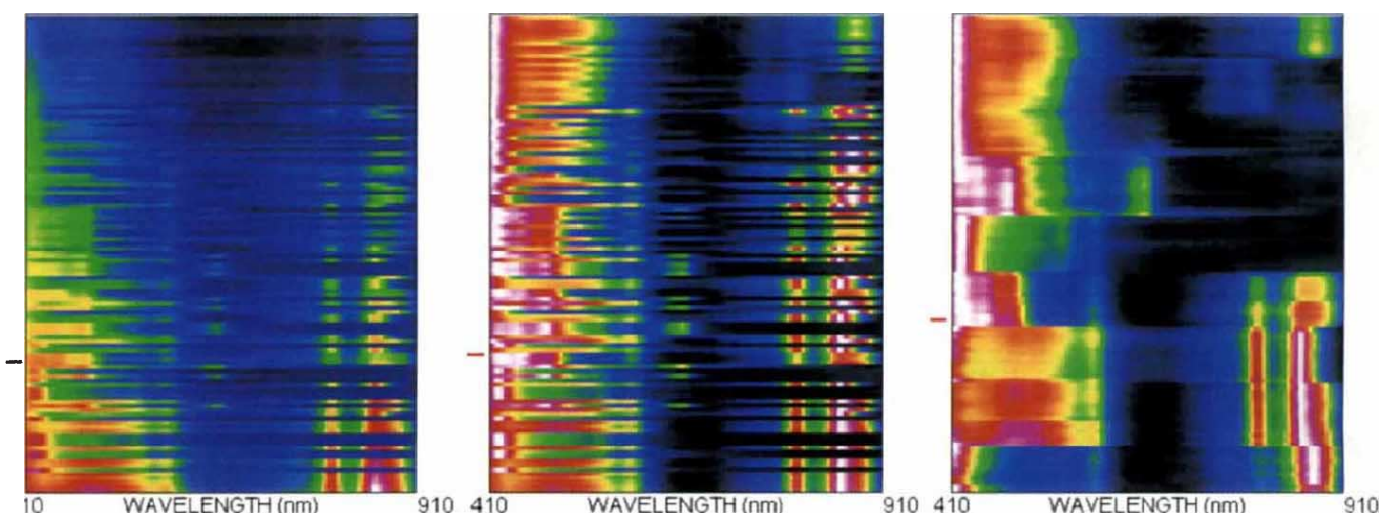


FIG. 2 After spectral sorting of the features shown in the previous figure, nine distinct classes of colonies are identifiable as groups of rows in the contour plot. The left panel shows the result of sorting these 112 spectra by the wavelength of maximum absorbance. The centre panel shows the result of rescaling these spectra per row, that is, the pseudocolouring scheme is recalculated to display the lowest-to-highest absorbance value in each feature. The right panel shows the result of a final 'similarity sort', which clearly demonstrates that nine different categories of strains are present. These rapid sorting techniques can be used to isolate unusual spectra that might otherwise not be seen.

tally surveyed by anything other than a sparse sampling.

One approach in searching such vast sequence spaces is to use exponential ensemble mutagenesis (EEM), a reiterative technique that increases the probability of finding diversely functional proteins. In EEM, libraries of smaller combinatorial groups of amino acids are initially generated using structural data, phylogenetic data or random mutagenesis (using an equiprobable mixture of G, A, T and C in all three codon positions). Functional mutants from each of these libraries are sequenced and a database of amino acids for each site is created. This information is combined and used for a second cycle of mutagenesis over a larger number of sites.

Formulating intelligent dopes

We have used computer algorithms to calculate the most optimal nucleotide mixture or 'intelligent dope' to construct each library. Optimal dopes encode a selected target set of amino acids while minimizing the nucleotide complexity. Using the three-dimensional genetic code in Fig. 3, one might decide to use (TCA)(CAG)(GATC) to encode the amino acids: lysine, asparagine, arginine, serine and threonine (that is, K, N, R, S and T). This mixture has a nucleotide complexity of $3 \times 3 \times 4 = 36$, and also encodes the amino acids: C, H, P, Q, W and Y. Using the group probability (P_G) error function and limiting the third codon to only G or C in the program CyberDope (KAIVOS, Inc., Mountain View, California), a less complex mixture can be found: (A)(CAG)(GC), complexity = 6. This dope would generate K(0.17), N(0.17), R(0.17), S(0.17) and T(0.33) where the number in parenthesis is the probability for the associated amino acid to occur at the site mutagenized. Since P_G is a measure of maximum entropy, the relative proportions of amino acids encoded by the

target set in this optimal dope are as close to equiprobable as possible given the limitations of current commercial DNA synthesizers. K, N, R, S and T are still encoded exclusively by (A)(CAG)(GTC), one of the next best dopes (slightly lower value of P_G). However, the relative frequencies of the amino acids are: K(0.11), N(0.22), R(0.11), S(0.22) and T(0.33). P_G calculations do not take into account the frequency of occurrence of amino acids in the target set as compared to a weighted sum-of-the-squares-of-the-differences (SSD) error function which is minimized by a best fit to the data. SSD omits infrequently used amino acids from the dope⁵⁻⁸.

Better antibodies

In a recent experiment combining the EEM strategy with a DIS assay, a 10^7 -fold advantage over random combinatorial

mutagenesis was achieved after mutagenizing 16 positions in a LH protein. Because most proteins are not coloured, it behoves us to look at some of the general features of combinatorial optimization that have been discovered in this model system. For example, the efficient mutagenesis of as many as 16 amino acid residues makes it feasible to mutate an entire CDR of a phage displayed antibody fragment (Fab)⁹. By combining sequence data from several low affinity (randomly mutagenized) antibodies, it is anticipated that a second cycle of mutagenesis (using a restricted set of amino acids at each sequence position) will result in a variety of tighter binding antibodies. This concept can be extended to other proteins for optimization of desired catalytic properties. □

Douglas C. Youvan is a senior investigator at the Palo Alto Institute for Molecular Medicine, a nonprofit research organization, 2462 Wyandotte Street, Mountain View, California 94043, USA. For further information on the reviewed products, fill in reader service number 100.

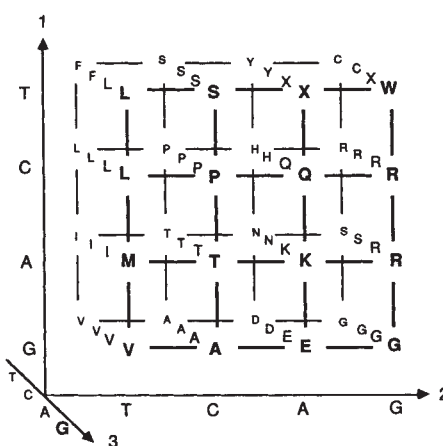


FIG. 3 A three-dimensional representation of the genetic code where the three axes correspond to the first, second, and 'wobble' position of the nucleotide triplet. Each of the 64 lattice points are occupied by the single letter representations of the encoded amino acids.

1. Yang, M. M. & Youvan, D. C. *Bio/Technology* **6**, 939-942 (1988).
2. Arkin, A. et al. *Bio/Technology* **8**, 746-749 (1990).
3. Youvan, D. C., Goldman, E. R., Delagrave, S. & Yang, M. M. *Meth. Enzym.* (in the press).
4. Yang, M. M. *Am. Biotech. Lab.* (in the press).
5. Goldman, E. R. & Youvan, D. C. *Bio/Technology* **10**, 1557-1561 (1992).
6. Delagrave, S., Goldman, E. R. & Youvan, D. C. *Protein Engng* **6**, 327-331 (1993).
7. Delagrave, S. & Youvan, D. C. *Bio/Technology* **11**, 1548-1552 (1993).
8. Arkin, A. P. & Youvan, D. C. *Bio/Technology* **10**, 297-300 (1992).
9. Barbas, C. F., Bain, J. D., Hoekstra, D. M. & Lerner, R. A. *Proc. natn. Acad. Sci. U.S.A.* **89**, 4457-4461 (1992).

Across the spectrum

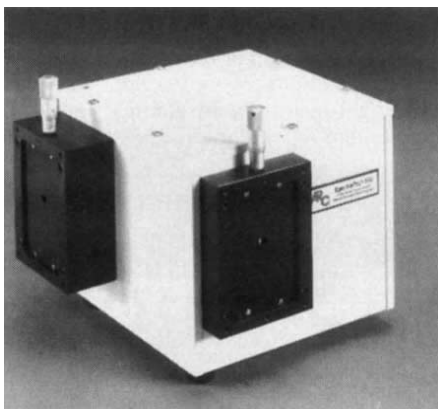
A cathodoluminescence imaging and spectrometry system, a benchtop mass spectrometer for gas analysis, an inductively coupled plasma/glow discharge mass spectrometer — new products from the spectroscopy marketplace.

MonoCL from Oxford Instruments is a **cathodoluminescence imaging and spectrometry system** designed to provide electron microscopists with the capability of detecting low intensity optical cathodoluminescence (CL) signals that are emitted by many materials when bombarded with an electron beam (*Reader Service No. 101*). The system can display panchromatic images at all wavelengths, as well as monochromatic images at single wavelengths. It is also designed to provide a high-resolution spectral analysis. MonoCL uses a high-throughput light collector and monochromator to provide a wide spectral range: from 160 nm in the ultraviolet up to 1,800 nm in the infrared. The company says that it also enables very low CL signals to be processed which, in turn, means low beam currents can be used. Design features include a photon counting capability, an integrated framestore and dedicated spectral analysis and archiving software. To provide the highest CL intensity, and the greatest spectral discrimination, MonoCL can be used with the company's helium- or nitrogen-cooled scanning electron microscope (SEM) stages. It can be interfaced to all SEMs, including field emission microscopes, together with some transmission electron microscopes.

Bruker Instruments announces the Advance series of **digital nuclear magnetic resonance spectrometers** for solids, liquids and imaging (*Reader Service No. 102*). A full range of configurations is available ranging from the very routine to the most demanding applications, including micro-imaging, CPMAS, wideline and CRAMPS. Systems are available from 200 to 750 MHz, with up to eight identical rf channels and up to two receivers. Advance systems are controlled by a UNIX workstation computer with X-11 Motif display environment. TCP/IP Ethernet is standard and optional process-

ing software is available for a range of workstation options, including Sun, SGI, IBM RISC, Macintosh and IBM-compatible PC systems. A range of probes is offered, including 2.5 mm micro-sample probes, 8-mm probes optimized for ^1H observation in low concentrations, and the new QXI probe for ^1H observation with three X-nucleus channels and a ^2D lock channel. With the appropriate automated sample changer, up to 120 liquid samples or 20 solid CPMAS samples may be analysed without operator intervention. DPX, DRX, DMX and DSX configurations are available in this series.

Princeton Instruments is distributing Acton Research's SpectraPro-150 **monochromator/spectrograph**, which features a high-throughput astigmatism-corrected imaging optical system for improved resolution when used as a spectrograph with a CCD detector (*Reader Service No. 103*). The astigmatism-corrected optical system is



SpectraPro-150 monochromator/spectrograph features an astigmatism-corrected imaging optical system.

said to deliver more photons per pixel than conventional spectrographs and as such is suitable for low light applications. Small size makes it suitable for interfacing to microscope systems. To allow the use of multiple inputs, the instrument has a large 10×25 mm focal plane. The SpectroPro-150 also features automated dual indexable gratings to allow users to cover a wider wavelength range at low resolution, and then to zoom in at higher resolution over a narrower wavelength range without having to remove gratings from the instrument. The dual grating turrets are kinematically mounted for easy interchange. The Auto-track electronic system keeps track of the gratings and provides the correct wavelength readout for any standard grating installed.

If portability is a plus then Abion offers the BioScan **portable fluorescence detector** which works in the 300 to 600 nm spectral range (*Reader Service No. 104*).



Abion's fluorescence detector for 'field' applications.

Stated sensitivities for ATP/luciferase tests is 1 nmol^{-1} and 10 ng l^{-1} for immunoassays with POD label. The device is supplied with an interchangeable measuring head for switching between fluorescence and luminescence measurement. Up to 5 h of remote operation is provided.

■ Spectrophotometers

The Polytec X-DAP is a **diode-array spectrophotometer** from Lambda Photometrics that uses an array of diode detectors, either 1,024 or 526 elements, replacing the single detector and scanning mechanism of traditional instruments (*Reader Service No. 105*). Each detector in the array sees a portion of the spectrum exiting from a dispersing prism and each produces a signal proportional to the intensity of the input at a particular wavelength. This way, a complete spectrum is collected simultaneously and the absence of moving parts is said to improve both accuracy and linearity. Three versions are available covering ultraviolet



Lambda's diode-array spectrophotometer.



Advance DPX spectrometer.

PRODUCT REVIEW

(UV), UV/visible and UV/visible/near-infrared. Typical integration time is stated as less than 25 ms and the spectral resolution is from less than 1.25 nm to less than 4.5 nm, depending on the wavelength range. The output signals from the detectors are digitized and the data are presented on a personal computer. XLAB software provides a range of control, data acquisition, storage, display analysis and calibration functions. A series of fibre-optic probes, cells and reflectance accessories permits a variety of analyses, both on and off-line, including colour measurements.

New from ATI Unicam are the UV3 and UV4 series of **ultraviolet/visible spectrometers** (*Reader Service No. 106*). The spectrometers feature master interferometric

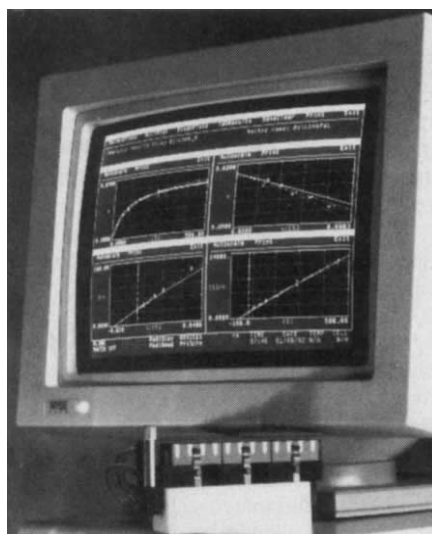


For the UV/visible range — see the UV3 and UV4 Series from ATI Unicam.

gratings that are designed to offer good stray light rejection. The double-beam optics utilizing the low inertia Hatten modulator are said to deliver a 5 ms measurement cycle. Other features are quartz-coated optics coupled with self-optimizing source alignment and a photomultiplier detector designed to ensure optimum signal-to-noise performance. The Intelliscan mode can be used for highly absorbing samples.

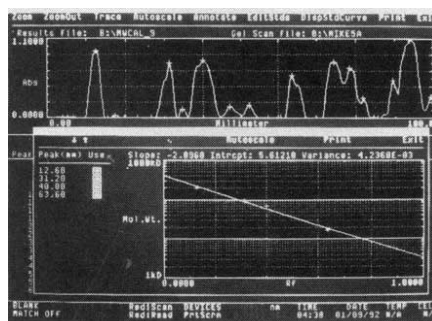
Jouan is eyeing medical and industrial applications for its VP 10.12 **visible spectrophotometer** (*Reader Service No. 107*). This lightweight unit is designed to be portable, with 12 V operation (transformer supplied) and carrying case for field operation. The sample compartment accepts cuvettes (including a funnel cell system) and 13 mm test tubes. For educational purposes, the instrument features an operating guide printed below the keyboard. Automation has been maximized with this model: calibration is automatic upon switch on, and keying in the required wavelength results in automatic movement of the monochromator followed by auto-zeroing.

Beckman offers assorted accessories and a software program for its DU Series 600 and 7000 ultraviolet/visible spectrophotometers (*Reader Service No. 108*). The integrated **enzyme mechanism and activity**



Beckman's integrated enzyme mechanism and activity software eliminates the need to transfer data to a PC or to use third-party software to perform an analysis

software automatically transforms data and calculates the enzyme parameters K_m , V_{max} , K_{cat} , K_i and the Hill coefficient. Michaelis-Menten, Lineweaver-Burk, Eadie-Hofstee, Hanes-Woolf and inhibitor plots are provided on the screen or the printer. There is no need to transfer data to a PC or use third-party software to perform the analysis. In the accessory arena, the **gel/film holder** is designed to move gel or film samples through the stable micro-focused beam. The integrated gel scan software automatically calculates and reports relative peak areas and



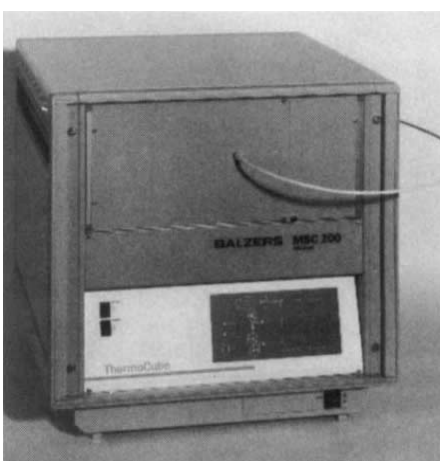
Gel and film scanning accessory for the DU Series 600 and 7000 UV/visible spectrophotometers.

individual components. It also allows the user to determine the molecular weight of samples based upon its generation of a standard curve of log molecular weight versus relative migration (R_f) distance. The molecular weight calibration curve is generated by the system from user standards and can be viewed and edited before and after use. The Micro-Auto 12 **cell holder** features water-regulated temperature control to measure samples at a given temperature required for enzymes and other temperature-sensitive samples. The cell holder allows the user to transfer up to 12 samples simultaneously from a 96-well microtitre plate into

Multi-Microcells using the company's 12-tip Multi-pipette or other commercially available products.

■ Mass spectrometers

Balzers has introduced the ThermoCube MSC200, a benchtop, computer-controlled **mass spectrometer for gas analysis** from 1–200 a.m.u. (*Reader Service No. 109*). A detection limit of less than 1 p.p.m. for non-interfering species and inert sampling line is designed to allow detection of condensable and reactive gases, making ThermoCube suitable for thermal analysis/mass spectrometry. The ThermoCube is powered by the company's interactive Quadstar 421 software package and operates under Windows 3.1. Analog signals from the thermobalance, such as temperature, weight loss and differ-



ential weight loss, are correlated with data from the mass spectrometer. Quadstar controls digital and analog inputs and outputs, allowing easy connection of the thermobalance to the ThermoCube. The instrument utilizes a quadrupole analyser, with a Faraday/Channeltron unit for long-term stability and low detection limits. The gas inlet system, made of inert materials, can be heated up to 200 °C. This, Balzers says, eliminates chemical reactions and condensations between the thermobalance and mass spectrometer.

An enhanced version of the Vision 2000 **laser desorption time-of-flight mass analyser** is now available from Finnigan MAT, which allows the user to work in a choice of operating modes including linear detector mode, post source decay (PSD) mode and reflector mode (*Reader Service No. 110*). Operation in linear or reflector modes provides complementary information on the same compound. Stepping of the reflector potential allows study of fragment ions, providing structural and sequence information on biopolymers. Quick switching between the three different operational modes is provided by the integrated instrument controller. Also look out for the Vision 2000L linear time-of-flight mass spectrometer for matrix-assisted laser desorption/ionization.

Derived from the Vision 2000, the Vision 2000L features an acceleration potential of up to 30 kV in combination with a new hybrid ion detection system, and an *x, y* controlled inlet probe for up to 28 samples. This system can be upgraded to the full Vision 2000 reflector time-of-flight system including PSD scan mode for the most demanding biopolymer sequencing applications. Finnigan MAT's newest instrument is the Element double focusing **inductively coupled plasma/glow discharge mass spectrometer**. The optional glow discharge ion source in the Element is designed to expand the range of applications from measuring aqueous solutions to direct analysis of solids.

New from Perkin-Elmer is the Optima 3000 XL **inductively-coupled plasma-optical emission spectrometer** (ICP-OES) (*Reader Service No. 111*). Based on the Optima 3000 ICP-OES, the new version's plasma has been turned 90°. This position is designed to allow for analyses at much lower detection limits and increase the throughput of samples. The enhanced version also features the original detector design and optical system that enables users to measure the spectral background and each analyte line simultaneously. It also includes more than 5,000 emission lines, providing the flexibility to select alternate interference-free lines. The instrument consists of an Eschelle-based polychromator with a segmented-array charge-coupled detector, a 40-MHz free-running RF generator and temperature-controlled plasma pneumatics. A 486-based computer controls the instrument and all instrument functions, including plasma operation, control of the autosampler and data acquisition.

Biflex is the new research-grade **matrix-assisted laser desorption/ionization time-of-flight mass spectrometer** from Bruker Instruments (*Reader Service No. 112*). The instrument is designed to provide structural and sequence information and MS/MS (tandem mass spectrometry) capabilities directly in a time-of-flight mass spectrometer. The system incorporates the FAST (fragmenta-

tion and structural time-of-flight-mass spectrometry) methodology. It is also a high-end matrix-assisted laser desorption/ionization time-of-flight mass spectrometer, designed to be capable of obtaining molecular ion masses from small to large molecules (up to several hundred thousand daltons) rapidly and with subpicomole sensitivities. Fragmentation and structural information can be provided for most peptides or lycopeptides up to 3 kilodaltons, and in some cases up to and beyond 10 kilodaltons, says Bruker. The instrument is intended to complement Edman analysers for sequence determination and identification and localization of post-translational modifications.

Fisons Instruments/VG BioTech recently launched the Platform-II **mass spectrometer for benchtop GC/LC/MS** to complement their dedicated Platform-I API LC/MS system (*Reader Service No. 113*). Both systems incorporate an updated data system, MassLynx-II, which provides data handling and analytical project management within a Microsoft Windows environment. Specially designed to suit the first-time MS user, this new system has been set up to accommodate both the established and most advanced ionization/sample introduction techniques. Features of the Platform-II include solids probe, DCI, GC/MS and particle beam LC/MS options for classical electron ionization analysis, providing library searchable mass spectra, as well as a differentially pumped vacuum system. Additionally, the system features an atmospheric pressure ionization LC/MS interface. APci and Megaflow electrospray inlet options allow the widest possible range of 'problem' analytes to be accessed by LC/MS. Also look out for the new MD 800 family from Fisons Instruments/VG MassLab, a series of instruments specially tailored to suit all benchtop GC/MS applications. Comprising five configurations in all, the MD 800 family is now available with both positive and negative ion chemical ionization. Additionally, all models can be upgraded.

■ Software

Hitachi Scientific Instruments has introduced a Windows-based software package for the **measurement of intracellular cation concentrations** using its F-4500 fluorescence spectrophotometer (*Reader Service No. 114*). The software provides control of the instrument, including automatic acquisition of time- and wavelength-based excitation/emission spectra and calculation of intracellular cation concentration and kinetics. A range of post-run processing facilities are available for the presentation of data. The F-4500 itself has a fast wavelength drive, giving a stated slew rate of 60,000 nm min⁻¹. The data interval for measurements can be as little as 0.3 s, Hitachi says. As calcium ion measurements are generally made at two wavelengths by monitoring the fluorescence from Fura-2 indicator both bound to Ca²⁺

and free from Ca²⁺, this means the complete measurement cycle takes 0.6 s. The system can accept up to eight sets of excitation and emission wavelengths, enabling measurements not only of calcium ion concentration but also of intracellular pH using BCECF as indicator and blood platelet aggregation using scattered excitation light to be made in a single experiment.

EDAX is making available an upgraded version of its **software package for use with the NX-2 (UNIX-based) microanalysis workstation** (*Reader Service No. 115*). The new version incorporates Sun OS 4.1.3 operating system, open Windows 3.0 graphic



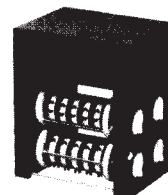
NX-2 UNIX-based microanalysis workstation — see EDAX.

environment, Island Graphics 4.0 desktop publishing and CD-ROM drive as standard. Its upgraded application software provides many enhancements including EDS identity, EDS display, EDS quantify, Linescan display, image display, X-ray image/Linescan acquire and ROI tools. □

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Bruker's Biflex MALDI-TOF mass spectrometer also provides structural and sequence information.

A twist in the tail of human endothelin

The X-ray crystal structure of endothelin reveals a peptide with a compact globular conformation that may well represent the active form of the molecule.

ENDOTHELIN, a small 22-amino-acid peptide produced by endothelial cells, was originally defined as one of the most potent vasoconstrictors of the smooth muscle sheath that lines the larger blood vessels. As such, endothelin is implicated in the regulation of vascular tone and has been suggested to be important in cardiovascular diseases such as hypertension, vasospasm and atherosclerosis. A paper in this month's *Nature Structural Biology* now presents the first crystal structure of endothelin, as determined by Janes and colleagues¹. Of the various endothelin structures that have been proposed, most based on NMR data, this new X-ray crystal structure is suggested to be the best candidate for the active, *in vivo* conformation of this important molecule.

The single layer of endothelial cells that lines our blood vessels has a more than purely mechanical role in the circulatory system. These cells, in response to various stimuli, produce factors that are involved in maintaining vascular homeostasis — endothelin and the endothelium-derived relaxing factor nitric oxide being among the best known.

There are three variants of endothelin (ET-1, ET-2 and ET-3), all of which are derived by proteolytic cleavage from a larger precursor protein. The small size of the endothelin peptide makes it an ideal candidate for structure determination by NMR. Nonetheless, there is no consensus conformation among the different solution structures, which may be due to the non-aqueous conditions used to obtain sufficiently high concentrations of peptide for analysis.

The X-ray crystal structure of endothelin, derived from aqueous solution, reveals that the peptide adopts a compact globular conformation. The N terminus forms an extended β -strand followed by a bulge and loop (residues 5–11) and the C-terminal portion of endothelin forms an

irregular α -helix (residues 12–21). The four cysteine residues — absolutely conserved between the endothelins, and the related sarafotoxins and bibrotoxin — form two disulphide bridges that hold the N-terminal strand close to the proximal part of the helix (see figure). Sequence conservation in the C-terminal region suggests that all three endothelins will form similar α -helical tails. Most sequence variability is seen in the bulge region of the structure.

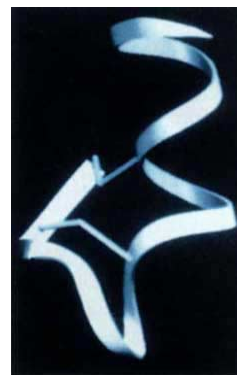
In the absence of a homologous crystal structure, various of the NMR models were used as search structures; the backbone of the N-terminal portion of one of the NMR models was sufficiently similar to the same portion of the peptide in the crystal structure to allow a molecular replacement solution; indeed, the root-mean-square deviation (r.m.s.d.) between the two structures in this region is 2.0 Å. But when all the sequences are included in the comparison, the r.m.s.d. shoots up to 5.0 Å, emphasizing the very different C-terminal conformations.

There are two classes of endothelin receptor (ET_A and ET_B) with different affinities for the different endothelins: ET_A has a similar affinity for ET-1 and ET-2 but a lower affinity for ET-3, whereas ET_B has comparable affinity for all three endothelins. For the ET_A receptor, the nature of the residue at position 2 of endothelin and the overall structure of the head group, residues 8–13, are important for binding.

For the ET_B receptor, on the other hand, determinants in the C-terminal tail are critical in the interaction. The α -helix itself may be important for ET_B receptor binding because the binding/activity is particularly sensitive to replacement of residues positioned on the same side of the helix.

The conformation of such a small peptide could well be influenced by packing interactions in the crystal. Janes *et al.* note that two intermolecular hydrogen bonds may distort the conformation of the head group, particularly in the vicinity of residues 8 and 13, but there are no close contacts to the backbone residues of the C-terminal helix, so crystal packing forces may not be responsible for the difference between the NMR and crystal structures in this region.

What relevance does the crystal structure of endothelin have to the biologically active conformation? The two disulphide bridges and the extensive network of



X-ray crystal structure of human endothelin¹ (reproduced from ref. 1 with permission). The alpha-carbon ribbon is shown with the two disulphide bridges linking the distorted α -helix with the N-terminal β -strand.

hydrogen bonds that stabilize the structure suggest that the peptide is unlikely to be very flexible and that the structure seen in the crystal may well represent the active conformation. Janes *et al.* point out that many of the invariant residues constitute the hydrophobic core of the structure and that variable residues can be accommodated without serious disruption of the structure.

What of the *in vivo* function of endothelin? Its role as a vasoconstrictor has recently been questioned by the finding that the blood pressure of transgenic 'knock-out' mice with only one copy of the gene, is slightly raised, rather than lowered as expected. Perhaps even more surprisingly, disruption of both copies of the gene results in an embryonic lethal phenotype with malformed craniofacial organs, which seems to originate from abnormalities of the pharyngeal arches².

The endothelin knock-out mice clearly indicate that our understanding of the function of this small peptide, both in the control of vascular tone and during embryonic development, is far from complete. The insights gleaned from the X-ray crystallographic study¹ should facilitate the design of reagents that modulate the activity of the endothelin receptors, the proteinases responsible for the processing reaction, and so on. Such reagents will be invaluable in further defining the complex role of endothelin *in vivo*. The next step is to determine the details of the interaction between peptide and receptor in the hope of bringing the structure closer towards what is still a somewhat elusive function.

Guy Riddihough

Guy Riddihough is Editor of *Nature Structural Biology*.

1. Janes, R.W., Peapus, D.H. & Wallace, B.A. *Nature struct. Biol.* **1**, 311–319 (1994).
2. Kurihara, Y. *et al.* *Nature* **368**, 703–710 (1994).

Also in this month's *Nature Structural Biology*: *in vitro* selection of an RNA that can bind a small aliphatic hydrophobe; a three-dimensional model of the Rev-binding element of HIV-1; a new graphical representation for the secondary and tertiary structure of group I introns; comparing interleukin-4 NMR and crystal structures; kinetic partitioning during protein folding; engineering an allosteric activation site in glycogen phosphorylase; and conformational analysis of protein side chains.